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YAP AND TAZ DRIVE MELANOMA THROUGH OVERLAPPING AND YAP SPECIFIC PATHWAYS

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Table of Contents

LIST OF FIGURES

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ABSTRACT

Melanoma is an aggressive tumor with a high degree of metastasis and limited therapeutic options. Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ-Binding Motif (TAZ) are transcriptional coregulators implicated in driving tumor progression and metastasis in a wide variety of cancers, including melanoma. While YAP and TAZ have historically been thought of to act in a redundant fashion, there is growing evidence from both developmental and cancer contexts that they may have both overlapping and unique functions of their own. As most prior studies on YAP and TAZ in melanoma have focused on both of the proteins simultaneously, it is currently unknown if YAP and TAZ are able to contribute to melanoma progression in unique fashions.

In this dissertation I explored how YAP and TAZ drive melanoma growth, metastasis, and migration. I first identify that inhibition of YAP, but not TAZ, induces morphological changes in melanoma cells. Further functional assays reveal that YAP, but not TAZ, inhibition reduces melanoma cell numbers, ability to invade into matrigel, and numbers of focal adhesions. Using a non-biased RNA-sequencing approach, I demonstrate that YAP and TAZ regulate different transcriptomes in melanoma. Further analysis of the RNA-sequencing results revealed ARP2/3 complex member ARPC5 as a YAP specific downstream gene that regulates focal adhesion numbers and migration in our melanoma cell lines. These results lead me to propose a model in which YAP uniquely drives melanoma migration and focal adhesion numbers through regulation of ARPC5.

As YAP and TAZ have been implicated to drive melanoma progression and metastasis, I also examined the effect that Verteporfin, a small molecule inhibitor that is actively used in the clinic for other purposes but was shown to inhibit YAP and TAZ transcriptional activity *in vitro*,

has on melanoma cell initiation and progression in a transgenic melanoma mouse model ($Braf^{CA}$; Tyr-CreERT2; $Pten^{f/f}$). While other groups found Verteporfin beneficial in the inhibition of tumor growth on xenograft models, I found no effect on melanoma initiation or progression in our transgenic mouse model using much lower, but clinically relevant, doses of Verteporfin. These results provide evidence that targeting both YAP and TAZ with Verteporfin may not be advantageous in melanoma. Additionally, these findings are significant and novel because it reveals that while higher doses of a small molecule inhibitor in xenograft models can seem to be a potential therapeutic, clinically relevant doses in model systems that better mirror disease progression may not have the same effect.

Taken altogether, my studies provide evidence that YAP, but not TAZ, uniquely regulates melanoma cell progression and survival. Small molecule inhibitors that are YAP specific have promise as a less toxic and more beneficial strategy for fighting melanoma as both YAP and TAZ have been implicated to play redundant roles in tissue homeostasis and stem cell maintenance. Furthermore, my studies demonstrating that YAP and TAZ have vastly different functions in melanoma provide a foundation for future studies into how YAP and TAZ can differentially regulate downstream pathways.

CHAPTER 1: INTRODUCTION

1.1 Overview

Over 20 years ago, a series of genetic screens identified a central core kinase cascade controlling organ size during development in *Drosophila melanogaster*. This molecular pathway was subsequently named the Hippo pathway, after one of the genes in the pathway (**Figure 1.1**). Since its initial discovery in *Drosophila*, the Hippo signaling pathway has since been shown to be evolutionary conserved from the common fruit fly to mammals. The *D. melanogaster* protein *Yorkie* functions as main effector of the Hippo pathway by acting as a transcriptional coactivator (J. Huang et al. 2005). In mammals there exists two homologs of *Yorkie*, Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ binding motif (TAZ). YAP and TAZ are considered paralogs with similarities in gene and protein structure, as well as in a number of key functions, including cell proliferation and migration. YAP and TAZ both share similar protein domains, leading to binding and interacting with many of the same partners (**Figure 1.2**). Furthermore, it has been demonstrated that both are regulated by the Hippo pathway in a similar manner (Hao et al. 2008; Meng et al. 2015).

Due to the Hippo pathway's regulation of cell growth and migration in development, it is not surprising that soon after the discovery of YAP and TAZ as the main effectors of the Hippo pathway, they were hypothesized to play roles in driving tumorigenesis and metastasis. Indeed, it has since been shown that YAP and TAZ are overexpressed in a variety of cancers, and are correlated with a poor prognosis (Menzel et al. 2014; Zanconato, Cordenonsi, and Piccolo 2016; Panciera et al. 2017; L. Wang et al. 2013; W. Kang et al. 2011; Hall et al. 2010; Ge et al. 2017).

Furthermore, the dysregulation of these transcriptional coactivators regulate different cellular processes in various cancers, including but not limited to proliferation, metastasis, and drug resistance (Lamar et al. 2012; Zanconato et al. 2015; Zanconato, Cordenonsi, and Piccolo 2016; Piccolo, Dupont, and Cordenonsi 2014; Varelas 2014). Due to their ability to regulate cancer invasion and metastasis, YAP and TAZ have emerged as potential drivers of the highly metastatic skin cancer, melanoma.

Melanoma is a cancer with an aggressive nature and a high degree of metastasis that tends to affect fair skinned individuals (Tas 2012). As a cancer with a rising incidence rate, cutaneous melanoma is the 5th most common cancer in men and women in the United States of America (Tas 2012; Siegel, Miller, and Jemal 2019). From 2009-2015, the 5 year survival rate for people diagnosed with melanoma is 92% for all cases, but only 25% for patients whose tumor had already metastasized (American Cancer Society 2019; Howlader N et al. 2019). While localized primary cutaneous melanoma is often excised through surgical methods, metastatic melanoma presents a clinical challenge with few therapeutic options and significant potential for morbidity and mortality. While research into how melanoma progression and metastasis occur has greatly advanced during the last decade, the elucidation of oncogenic factors and molecular pathways are still crucial for future melanoma therapeutics. YAP and TAZ are known to drive invasion and metastasis in other cancers, but research into their role in driving melanoma metastasis is relatively limited.

Since YAP and TAZ are both regulated by the Hippo pathway, contain similar structural elements, and only have one constituent present in *Drosophila*, they have traditionally been considered to have overlapping and redundant functions. Despite this, recent studies indicate that

YAP and TAZ contain diverse functions from one another, and that these key differences drive distinct cellular processes in a cell. As YAP and TAZ have been implicated in actively driving progression of various cancers in cell type specific manners, it is important to treat the two transcriptional coactivators as different proteins.

In this introductory chapter, I first review the regulation of YAP and TAZ in terms of Hippo pathway dependent and independent factors, as well as in terms of binding partners interacting through conserved domains, with an emphasis on known similarities and differences between the two paralogs. I then provide an overview of the overlapping and unique roles that YAP and TAZ play in melanocyte development, mature melanocyte maintenance, and in the genesis and progression of melanoma. Due to the wide range of cellular functions that YAP and TAZ regulates, decoding how these factors work in parallel or uniquely will provide a greater understanding of melanoma initiation, metastasis, and potential treatment.

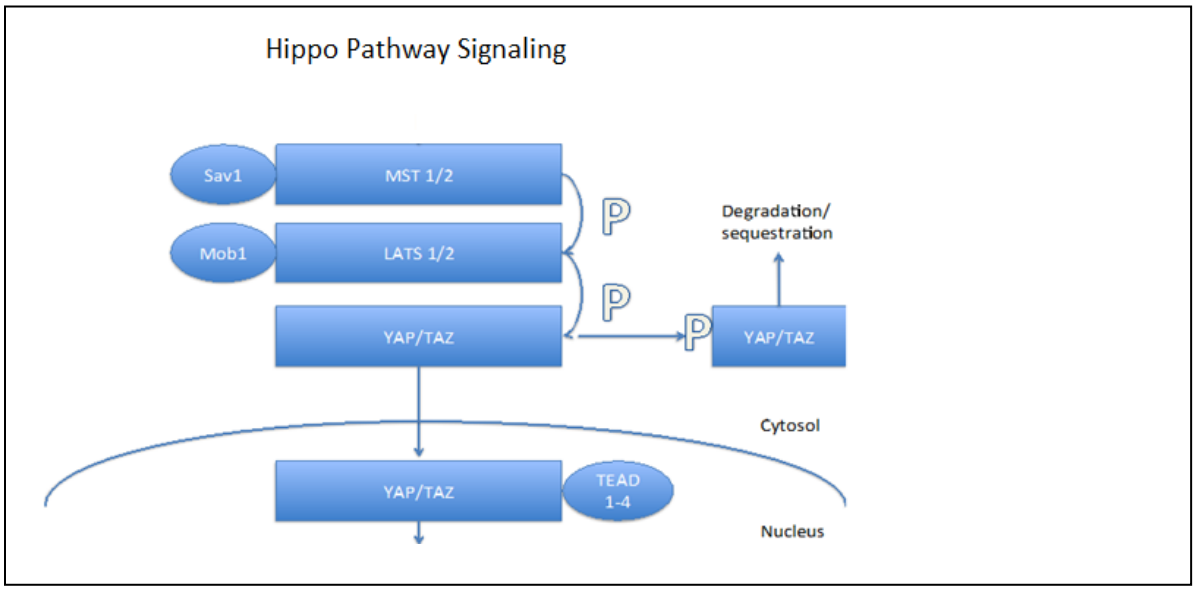

Figure 1.1: The Hippo Signaling Pathway and its major regulators in mammals.

Figure 1.1 (continued) Model of Hippo Pathway in mammals. The active Hippo pathway induces Mammalian Sterile 20-like kinases 1 and 2 (MST1/2) complex with Salvador homolog 1 (SAV1) to phosphorylate large tumor suppressor 1 and 2 (LATS1/2) in complex with MOB kinase activator 1A and 1B (MOB1A/MOB1B), which then phosphorylates YAP and TAZ at specific serine residues. Phosphorylated YAP and TAZ are retained in the cytoplasm via binding to 14-3-3 proteins or targeted for degradation. A lack of phosphorylation allows YAP and TAZ to translocate into the nucleus, where they bind the Transcriptional Enhanced Associate Domain (TEAD) family of proteins to drive transcription of target genes.

1.2 Regulation of YAP and TAZ

1.2.1 The Canonical Hippo Signaling Pathway

A major foundation on the regulation and functions of transcriptional coactivators YAP and TAZ is derived from *D. melanogaster* research, where the homologous coactivator *Yorkie* was shown to act as the main nuclear effector of the Hippo pathway (J. Huang et al. 2005).

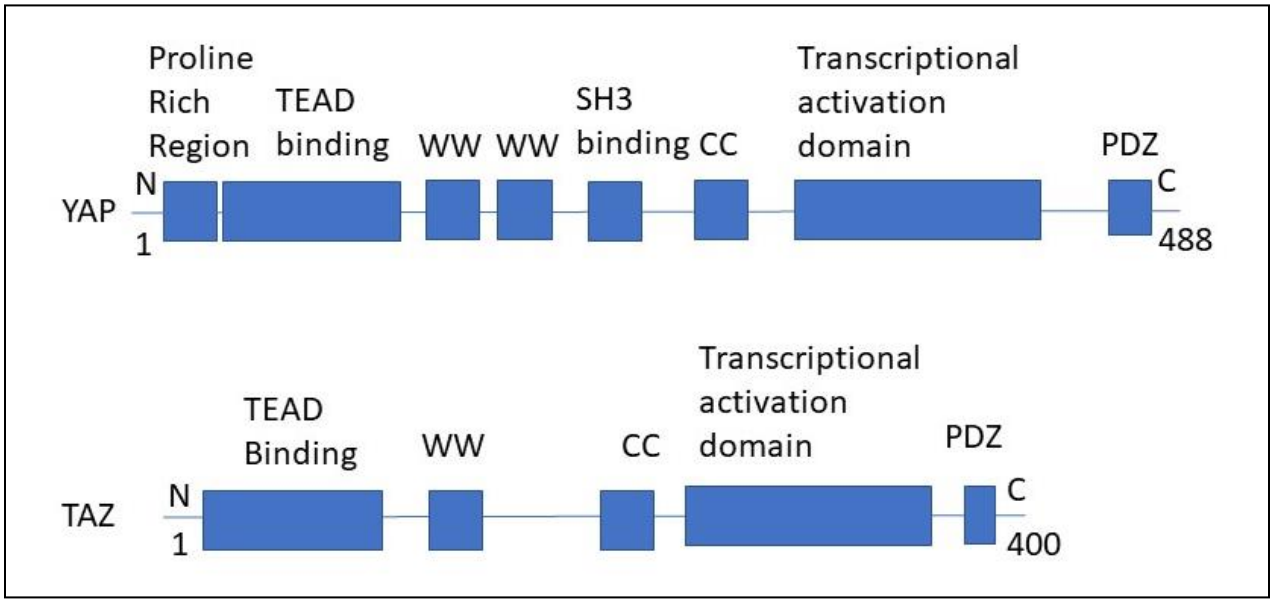

Figure 1.2: Structural Representation of protein domains found in YAP and TAZ. Regulatory elements found in YAP and TAZ. Domains found on both coactivators include the

Figure 1.2 (continued) TEAD binding domain, the WW domain, transcriptional activation domain, and a PDZ binding motif. Unique to YAP is the SH3 binding element, an extra WW domain, and a proline rich region.

As this chapter has an emphasis on cancer and melanoma, we will largely focus on the mammalian homologs. The Hippo pathway core kinase cascade consists of Mammalian Sterile 20-like kinases 1 and 2 (MST1/2) complexing with Salvador homolog 1 (SAV1) to phosphorylate large tumor suppressor 1 and 2 (LATS1/2) in complex with MOB kinase activator 1A and 1B (MOB1A/MOB1B), which then phosphorylates YAP or TAZ at specific serine residues (Zanconato, Cordenonsi, and Piccolo 2016; Varelas 2014). Upon phosphorylation, YAP and TAZ are then bound by 14-3-3 protein and retained in the cytoplasm. A lack of phosphorylation allows YAP and TAZ to translocate into the nucleus, where they bind transcription factors to drive transcription of target genes. A model of the core kinase cascade and its regulation of YAP and TAZ is detailed in **Figure 1.1**. Since the initial elucidation of the transcriptional coactivators YAP and TAZ and their regulation by the Hippo pathway in development, the downstream effectors YAP and TAZ have been shown to actively regulate expression of genes essential for tumor initiation, survival, and metastasis.

1.2.2 Non Canonical Hippo signaling pathways

Since the discovery of the Hippo pathway and its downstream effectors YAP and TAZ, numerous studies have discovered other molecular pathways that regulate YAP and TAZ independent of the canonical Hippo pathway. Increasing evidence reveals that various other factors can influence YAP and TAZ localization, function, and stability. Other pathways that

interact with YAP and TAZ include Wnt signaling and G-protein receptor signaling. Furthermore, external mechanical forces can exert extrinsic signals that influence YAP and TAZ activity, in part due to their function in development in regulating organ size and shape (Varelas 2014; Dobrokhotov et al. 2018; Low et al. 2014). Taken together, it is evident YAP and TAZ are regulated by a variety of molecular pathways and external signals besides the canonical Hippo pathway, either acting in tandem with constituents of the Hippo pathway, or through Hippo-independent mechanisms.

YAP and TAZ interact with both the canonical and non-canonical Wnt pathways. With canonical Wnt pathway signaling, a Wnt protein ligand binds to Wnt receptors at the cell surface which then induces an accumulation of β-catenin in the cell and nucleus where it helps to facilitate Wnt downstream transcriptional activity to regulate various cell processes, including proliferation and migration. Numerous studies have shown that misregulation of the Wnt pathway or mutations to key Wnt pathway constituents promote tumorigenesis (Howe and Brown 2004; Duchartre, Kim, and Kahn 2016; Paluszczak 2020; Kotelevets and Chastre 2020; You et al. 2020; Lustig and Behrens 2003). Cross talk between Wnt/β-catenin and YAP/TAZ activity play strong roles in promoting tumorigenesis. For example, the β-catenin destruction complex, a key component of canonical Wnt signaling, has been found to sequester YAP and TAZ in the cytoplasm in HEK-293 cells which limits the transcriptional activity of YAP and TAZ in the nucleus (Azzolin et al. 2014). Furthermore, the same research group found that the presence of YAP and TAZ are essential for the β-catenin destruction complex to actively degrade β-catenin. Outside of canonical Wnt signaling, Wnt based signaling has also been shown to work through alternative “non canonical” pathways that regulate planar cell polarity and intracellular

calcium levels in bone marrow-derived mesenchymal stem cells. These non canonical pathways were demonstrated to activate YAP and TAZ activity through regulation of YAP/TAZ nuclear localization to induce osteogenic differentiation and cell migration (Park et al. 2015).

Despite many of these studies elucidating non biased regulation of YAP and TAZ, there is strong evidence for the unique regulation of YAP or TAZ in other contexts. TAZ but not YAP contains a N-terminal phosphodegron that is targeted by GSK3, a component of the Wnt pathway (W. Huang et al. 2012). Disheveled (DVL), another key player in Wnt signaling, binds YAP to facilitate its nuclear transport in a YAP-phosphorylation dependent manner in MCF-7 breast cancer cells (Y. Lee et al. 2018). DVL interaction with TAZ through the carboxy terminal PDZ-binding motif and WW domain in MDA-MB-231 breast cancer cells inhibited downstream Wnt pathway activity (Varelas, Miller, et al. 2010). Interestingly, Wnt signaling directly activates gene expression of YAP to drive colorectal carcinoma cell growth in colorectal carcinoma cells (Konsavage et al. 2012). In melanoma associated fibroblasts, inhibition of Wnt signaling inhibits nuclear translocation of YAP (T. Liu et al. 2019). It was demonstrated that this inhibition of YAP activity led to a suppression of extracellular matrix remodeling and subsequent tumorigenic activity. These studies, among others, suggest cell type specific cross talk between the Wnt pathway and regulation of YAP, TAZ, or both YAP and TAZ. As transcriptional coactivators that function transcriptionally through their binding partners, it is possible that they play vastly different roles in different cell types, due to differences in the expression patterns of their binding partners as opposed to modifications to expression levels of YAP or TAZ. Furthermore, the tight integration between YAP, TAZ, and Wnt signaling reveals various pathways that cancer cells can hijack to regulate YAP and TAZ downstream activity.

YAP and TAZ are regulated by G-protein coupled receptors (GPCRs) through changes to YAP and TAZ subcellular location or activity through modulation of HIPPO pathway mediated phosphorylation. GPCRs represent the largest family of cell surface receptors (Luo and Yu 2019). They serve to mediate cellular responses to a wide variety of external signals and are implicated in driving human cancers through downstream regulation of cell proliferation, invasion, growth, and survival (Arakaki, Pan, and Trejo 2018; S. et al. 2018; Y. H. Zhang et al. 2020; Feng et al. 2014; Kuzumaki et al. 2012; Chua et al. 2017). Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are molecules that drive cancer progression by signaling through GPCRs to exert their downstream effects. Both molecules regulate YAP through control of YAP subcellular localization (Miller et al. 2012). S1P induces cell proliferation in hepatocarcinoma cells through its control of YAP activity while LPA activates nuclear YAP and TAZ activity to induce cellular migration and invasion in ovarian cancer cells (Cheng et al. 2018; Cai and Xu 2013). Both LPA and S1P regulate YAP and TAZ activity through inhibition of Hippo pathway component LATS1/2 kinases (Yu et al. 2012). LPA and S1P are not the only ligands that alter YAP and TAZ activity through GPCR binding. Depending on what GPCR the ligand is working through, YAP and TAZ activity can be increased or decreased through regulation of the Hippo pathway. Hormones epinephrine and glucagon binding to their respective GPCRs inhibited YAP/TAZ activity through activation of LATS1/2 kinases (Yu et al. 2012). In breast cancer cells, catecholamine, another naturally produced hormone that functions through GPCRs, inhibits YAP and TAZ activity (Dethlefsen et al. 2017).

While most of these studies show similar impact on YAP and TAZ activity through GPCRs and subsequent regulation of Hippo pathway members, there is evidence that suggests

differences in the regulation of YAP and TAZ. LPA treatment leading to YAP and TAZ dephosphorylation and subsequent nuclear activity resulted in different time frames. The LPA induced dephosphorylation on YAP peaked at 2 hours, while the effect continued on TAZ after 4 hours. Direct treatment with the catecholamines epinephrine and norepinephrine in MCF-7 breast cancer cells induced YAP phosphorylation as soon as 15 minutes post treatment (with no change in YAP protein levels), whereas the treated cells exhibited reduced TAZ protein levels after 30 minutes and up to 2 hours (Dethlefsen et al. 2017). These examples of differences in timing could be important for these cancer cells, where the cell needs to regulate YAP more quickly than TAZ to drive YAP specific processes to survive (Cai and Xu 2013). This difference in timing, along with the diversity of the GPCR family, provides any given cell type a number of options with which to regulate YAP, TAZ, or both YAP/TAZ activity.

Initial studies examining extrinsic signals affecting YAP and TAZ activity found that mechanical inputs strongly influenced the localization of these transcriptional cofactors. Contact inhibition through cell-cell contact, extracellular matrix remodeling, and cell shape are just several of the mechanical inputs that affect YAP and TAZ localization/activity (Zhao et al. 2007; Halder, Dupont, and Piccolo 2012). Contact inhibition blocks translocation of YAP and TAZ to the nucleus. However, prostate and liver cancer cells can overcome contact inhibition through increased YAP activity. In turn, inhibition of YAP rescued contact inhibition in cancer cells (Zhao et al. 2007). This was attributed to direct regulation of the Hippo pathway by tumor suppressors FAT and NF2, key players in mediating contact inhibition. Independent of the Hippo pathway, a stiffer extracellular cellular matrix (ECM) can regulate YAP and TAZ activation through integrin signaling and subsequent Rho GTPase activity (Dupont et al. 2011; Low et al.

2014; Dupont 2016). Modifications to the ECM have long been thought to play crucial roles in cancer progression and metastasis (Jaalouk and Lammerding 2009; Montagner and Dupont 2020). As aberrant expression of YAP and TAZ have been shown in a variety of cancers, it is not surprising that multiple cancers have an ability to hijack control of the ECM to drive progression and metastasis. Breast cancers with increased ECM stiffness correlate with increased invasion (Acerbi et al. 2015). Furthermore, YAP transcription driven remodeling of the ECM promotes cancer cell invasion (Calvo et al. 2013). In liver cancer, the mechanotranducer molecule Agrin activates YAP and TAZ activity in stiffer ECM settings through inhibition of the Hippo pathway (Chakraborty et al. 2017).

Similar to Wnt and GPCR signaling, there is evidence for the unique regulation of YAP or TAZ function. In melanoma associated fibroblasts, YAP activity increased matrix remodeling, providing a feed forward loop for cancer progression and metastasis (T. Liu et al. 2019). Conversely, only TAZ is able to drive expression of ITGAV, an integrin factor essential for transmitting signals from the ECM into the cell in hepatocarcinoma cells (Weiler et al. 2020). What is noteworthy in both these situations is that YAP or TAZ drive changes to ECM downstream activity, which then increases YAP and TAZ downstream activity. It is possible that cancers take advantage of unique regulation of YAP or TAZ in feed forward loops that can additionally affect not only regulation of itself, but also its paralog.

These studies, among many others, conclusively show that regulation of YAP and TAZ can be varied and cell type specific. Furthermore, while many of these pathways exhibit dual YAP/TAZ regulation, there are examples of specific regulation of either YAP or TAZ in several different contexts, including those involving cancer progression and survival. These multiple

levels of overlapping and unique YAP and TAZ regulation provide more molecular pathways for cancer cells to hijack to control YAP and TAZ transcriptional activity. Further studies into unique regulation of YAP and/or TAZ could provide better accuracy in developing potential cancer specific therapeutics.

1.3 Structure of YAP and TAZ

YAP and TAZ are considered gene paralogs and have similarities with homologs in other organisms that only possess one YAP/TAZ constituent, such as the fly protein Yorkie (J. Huang et al. 2005). As paralogs, it is not surprising that the two transcriptional coactivators share 60% protein sequence and are regulated by and bind to many of the same proteins. (Plouffe et al. 2018; W. Hong and Guan 2012). As they do not contain DNA binding domains, binding partners are essential for YAP or TAZ transcriptional activity. Although there are many overlapping domains between the two, there exist domains unique to either YAP or TAZ. These TAZ-shared and unique structures in YAP include a TEAD binding domain, transcriptional activation domain (TAD), a coiled-coil (CC) motif, a PDZ region, a WW (tryptophan-tryptophan) domain, Src Homology 3 (SH3) binding domain, and a proline rich region. Insight into the functions of these unique domains could elucidate how YAP or TAZ could be driving specific functions (**Figure 1.2**). Here, we will first review the overlapping domains that YAP and TAZ share, followed by unique domains that each of the transcriptional coactivators contain, and lastly discuss how these domains could be contributing to exclusive YAP or TAZ function and or regulation.

Both YAP and TAZ contain a TEAD binding domain. The Transcriptional Enhanced Associate Domain (TEAD) family of proteins are often thought of as the main transcription factor binding partners for YAP and TAZ. In humans, there are 4 TEADs (TEAD1-4) that all contain a conserved N-terminal TEA DNA binding domain and a YAP/TAZ binding domain (Holden and Cunningham 2018; Pobbati and Hong 2013). Much of YAP and TAZ transcriptional activity in development and disease is attributed to its binding to TEAD to regulate gene expression. Overexpression of a mutant TEAD2 lacking the DNA binding domain reduced YAP dependent murine liver overgrowth (Liu-Chittenden et al. 2012), while TEAD4 mutants reduced cancer cell growth and colony formation in gastric cancer cells (Z. Shi et al. 2017). Mutations to the key serine residue in YAP's TEAD binding domain (S94) reduced YAP induced cell growth and epithelial-mesenchymal transition (EMT) (Zhao et al. 2008), while mutations to the analogous residue in TAZ's TEAD binding domain (S51A) also reduced TAZ induced cell growth and EMT (Heng Zhang et al. 2009). While evidence supports that TEADs are the main driving factor of YAP/TAZ driven cancer progression, they are not the only inducers. ChIP-seq analysis of MDA-MB-231 breast cancer cells found that 78 % of TEAD4 occupancy peaks co-occupied with YAP/TAZ peaks while ChIP-seq analysis of HuCCT Cc1p1 cholangiocarcinoma cells found that YAP peaks only overlapped with common TEAD1/4 sites 7% of the time (Zanconato et al. 2015; Galli et al. 2015). Similar to upstream regulation of YAP and TAZ, how dependent YAP and TAZ are on TEAD interaction and subsequent downstream function appears to be cell type and context dependent. It is possible that YAP and TAZ could induce expression of similar or unique transcriptomes depending on the presence or absence of

TEADs. Taken together, these results indicate that YAP and TAZ interact with TEADs through their TEAD binding domain to drive a large portion of their downstream oncogenic activity.

The interaction with TEADs is the most studied YAP/TAZ-related protein complex, and for good reason; a significant portion of YAP and TAZ target genes are TEAD-dependent, many of the target genes of this complex are important for normal organ development, and this interaction is correlated with aggressive cancer progression (Galli et al. 2015; Zanconato et al. 2015; Heng Zhang et al. 2009; Zhao et al. 2008). As increasing evidence reveal that the YAP/TAZ-TEAD interaction drives a variety of cancers, the potential of this complex as a therapeutic target rises. Vestigial Like Family Member 4 (VGLL4) is a tumor suppressor whose lower expression correlates with lower survival in a variety of cancers (Deng and Fang 2018). VGLL4 binds TEAD proteins, acting as a competitor for both YAP and TAZ. Overexpression of VGLL4 in a murine lung cancer model inhibited cancer growth and progression (W. Zhang et al. 2014), while similar results were shown in breast cancer studies (Y. Zhang et al. 2017). This presents a potential therapeutic avenue, where elucidation of the binding mechanism of VGLL4 to TEAD could act as a competitor for the oncogenic activity of YAP and TAZ through TEAD. Indeed, initial results have proven this to be a potential therapeutic. Molecules mimicking VGLL4 binding have been shown to inhibit colorectal and gastric cancer progression in mouse models (Jiao et al. 2014; 2017).

Another overlapping domain between YAP and TAZ is the PDZ binding domain. The PDZ binding domain facilitates binding to PDZ domains on other proteins to regulate a variety of biological functions (H. J. Lee and Zheng 2010). For both YAP and TAZ, the PDZ binding motif is crucial for their cytoplasmic localization through interaction with tight junction proteins

zona occludens-1 (ZO-1) and 2 (ZO-2) (Remue et al. 2010; Oka et al. 2010; Oka and Sudol 2009). Monomethylation by Set7 lysine methyltransferase promoted the cytoplasmic retention of YAP in mice embryonic fibroblasts (Oudhoff et al. 2013). Furthermore, it was suggested that monomethylation at lysine 494, being proximal to the PDZ binding domain, inhibited its binding to ZO-2 and subsequent translocation into the nucleus. This finding potentially adds an extra layer of regulation to YAP but not TAZ. In terms of carcinogenesis, loss of the PDZ binding motif (5SA) in YAP inhibited nuclear localization and subsequent YAP transcriptional activity (Shimomura et al. 2014). Taken together, the PDZ binding motif is crucial for both YAP and TAZ localization to the nucleus.

Located in the C-terminus of both YAP and TAZ is the transcriptional activation domain (TAD). This domain contains residues that impact YAP and or TAZ activity dependent on phosphorylation status. YAP tyrosine phosphorylation by Src/Yes tyrosine kinases promote the association of YAP with the RUNX2 transcription factor. Inhibition of phosphorylation results in YAP dissociation from RUNX2 and reduced RUNX2 transcriptional activity in osteoblasts (Zaidi et al. 2004). Furthermore, it has been suggested that Src activity is required for YAP activation in cancer associated fibroblasts (Calvo et al. 2013). In colon cancer cells, Src kinase phosphorylation on YAP regulates the complex formation of YAP, transcription factor TBX4, and β-catenin to drive antiapoptotic genes BCL2L1 and BIRC5, and that inhibition of YES1 led to a decrease of cancer progression in a murine model (Rosenbluh et al. 2012). c-ABL tyrosine kinase phosphorylation of YAP (Y357) occurs during DNA damage response in HEK293 cells, and that this phosphorylation increased association with p73 to drive transcription of proapoptotic genes (Levy et al. 2008). In turn, TAZ phosphorylation (Y316) by c-ABL drove

interaction with nuclear factor of activated T cells (NFAT5) to suppress NFAT5 transcriptional activity in response to hyperosmotic stress in renal cell lines. While there is a scarcity of research into how the TAD domain can contribute to tumorigenesis, what is evident from these studies is that the TAD contains residues in YAP and TAZ that can be phosphorylated in similar fashions to drive a variety of downstream transcriptional effects. Whether YAP and TAZ drive cancer various cancers hijack these mechanisms or not is currently unknown.

Within the transcriptional activation domain for both YAP and TAZ is the coiled coil (CC) domain. Often overlooked in YAP and TAZ biology, this domain is found in a wide variety of organisms from bacteria to plants to animals, and is a common domain found in many cellular proteins (Truebestein and Leonard 2016). CC domains act as molecular spacers, tethers, and scaffolds in a variety of different types of proteins, including transcription factors where the CC moiety assists in protein-DNA interactions. In addition, CC domains are also found in structural proteins, motor proteins, and a number of other cytoskeletal factors. Overall, a main function of the domain is to facilitate protein-protein interactions and to promote complexes with other molecules. Few studies have focused on the CC domain in YAP and TAZ, but there exists some evidence of differential activity due to differences in the CC domains between the two transcriptional coactivators. The CC domain of TAZ was demonstrated to be essential for binding with SMAD 2/3-4 complexes (Varelas et al. 2008). Crystal structure analysis revealed that while YAP and TAZ can heterodimerize, TAZ can additionally homodimerize, thanks to its coiled coil domain (Kristal Kaan et al. 2017). The authors further show that this allows the formation of a heterotetramer with TEADs in a TAZ, but not YAP, specific manner. This indicates a possibility that the CC domain is mediating TAZ specific processes. Even more

telling is that a deletion of the TAZ CC domain or replacement with YAP's CC domain reduced the ability of TAZ to drive downstream transcription in MCF-10A cells (Lu et al. 2020). They further demonstrate that the CC domain in TAZ is essential for TAZ driven compartmentalization of coactivators TEAD4, BRD4, and MED1 along with transcription elongation factor CDK9 and that this entire process was Hippo dependent. These studies provide evidence that the CC domains between YAP and TAZ have differential functions and that this can all be driven by the Hippo pathway, a regulatory pathway that is thought of as regulating YAP and TAZ similarly.

The WW domain is a small domain that mediates protein protein interaction. Generally, 5 classes of WW exist, depending on which motif they bind to (Kasanov et al. 2001).The WW domains in YAP and TAZ are classified as a Class I WW domain, as they bind to proline rich domains containing a consensus PPxY binding sequence (Chen and Sudol 1995; Macias et al. 1996). It is called the WW domain due to its characteristic double tryptophan residues that are usually spaced 20-22 bases apart (Bork and Sudol 1994). The WW domain in YAP and TAZ facilitates interactions with various proteins, including Hippo pathway effector LATS1/2 (Kanai et al. 2000; Hao et al. 2008). While both YAP and TAZ contain WW domains, YAP isoforms can contain either 1 or 2 WW domains, whereas TAZ only has 1 (Webb et al. 2011). The tandem WW domains act in a synergistic manner to facilitate ligand binding (Kanelis et al. 1998; X. Huang et al. 2009; Fedoroff et al. 2004). YAP isoforms with tandem WW domains bind dual PPxY containing peptides at an affinity 6-fold higher than isoforms containing one WW domain (Webb et al. 2011). The tandem WW domains in YAP are essential for reading and binding to a phospho-serine code found on activated SMAD 1/5 proteins to drive downstream BMP pathway

transcriptional activity (Aragón et al. 2011). While the first WW domain selectively mediated interaction with SMAD7, both WW domains were essential for interaction with SMAD1, where the first WW domain interacts with the PPXY site on SMAD1 and the second interacts with a phosphorylation dependent serine-proline motif (Aragón et al. 2012). This selectivity involving sequential binding to both WW domains reveals a potential YAP specific role in driving various cell processes. Thus, there is strong evidence that the tandem WW domains found in YAP and not in TAZ could bind ligands more strongly and subsequently drive different pathways to exert a YAP unique downstream affect. It is unknown if cancers take advantage of this difference in binding affinity but it is worth exploring.

While YAP and TAZ have several domains that have shared features and may have the same or similar function, two regions are uniquely found in YAP: the Src Homology 3 (SH3) binding domain and a proline-rich N-terminus. The SH3 binding epitopes mediates interaction with an approximately sixty amino acid SH3 moiety, which are often found on proteins involved in cell proliferation, migration, and cytoskeletal modifications (Kurochkina and Guha 2013). YAP and its SH3 binding peptide was first discovered to bind to Src/Yes tyrosine kinases, as well as adaptor proteins (Sudol 1994). In melanoma, proto oncogene SRC tyrosine kinase drives YAP/TAZ-TEAD transcriptional activity (Lamar et al. 2019). As SRC tyrosine kinase contains an SH3 domain, it is possible that regulation of YAP and TAZ activity by SRC could be accomplished with different mechanisms, as YAP or TAZ localization and ability to directly interact with SRC were not examined. While little studies on the significance on this domain have been conducted, it is possible that this binding domain plays a large role in YAP specific processes, as TAZ does not contain a SH3 binding domain. Another domain unique to YAP is

the proline rich region found at the N-terminus. This proline rich region has been shown to facilitate interaction with heterogeneous nuclear ribonuclear protein U (hnRNPU) in the nucleus to drive downstream expression (Howell, Borchers, and Milgram 2004). Much like the SH3 binding domain, very little research has focused on the significance of this region but it is entirely plausible that this proline rich region helps to drive YAP specific processes in various settings. While the shared domains of YAP and TAZ have overlapping and possible divergent functions, the presence of the YAP-specific SH3-binding peptide region and the proline rich region support that YAP has unique functions from TAZ.

1.4 A role for YAP and TAZ in melanoma precursor cells: embryonic neural crest and melanocytes

Melanoma cells are derived from pigment cells called melanocytes, which originate from the embryonic cell type called the neural crest. The neural crest is a transient population of migratory cells unique in vertebrate development that originate from an outgrowth of cells between the neural ectoderm and the neural tube (Cichorek et al. 2013; Saleem 2019). During embryogenesis, the multipotent neural crest cells migrate to specific anatomical locations to differentiate into a wide variety of cell types including peripheral and enteric neurons and glia, smooth muscle, craniofracial bone, and pigment cells (Knecht and Bronner-Fraser 2002; Cichorek et al. 2013; Uong and Zon 2010). Induction, migration, and differentiation of neural crest cells is all accomplished through a complex network of different levels of BMP, Wnt, Notch, and FGF signaling that works in combination with internal cellular cues from

transcription factors to provide the instructions needed for proper differentiation and migration of neural crest cells (Cichorek et al. 2013; Saleem 2019). YAP and TAZ were discovered to be both necessary and sufficient for neural crest migration, although the majority of the studies purely focused on YAP. YAP expression promotes neural crest migration in zebrafish, mouse and chicken embryos, as well as neural crest cells in culture (Kumar, Nitzan, and Kalcheim 2019; J. Wang et al. 2016; Hindley et al. 2016; Dooley et al. 2019; Bhattacharya, Azambuja, and Simoes-Costa 2020). The expression and localization of activated YAP within the cell nucleus precedes migration as well as the activation of the genetic pathways driving this process (Bhattacharya, Azambuja, and Simoes-Costa 2020; Kumar, Nitzan, and Kalcheim 2019). Co-electroporation of a shYAP construct and a BMP reporter containing BMP response elements upstream of GFP into the dorsal neural tube of avian embryos revealed a loss of BMP activity in premigratory and emigrating neural crest cells in the presence of YAP inhibition. Similar results were found using a Wnt-GFP reporter in the presence of YAP inhibition. This revealed that YAP is both necessary and sufficient for the early activity of both BMPs and Wnts in the pre-migratory crest, two pathways that are key for neural crest migration (Kumar et al. 2019; Kléber et al. 2005). Interestingly, the function of YAP is also dependent on both the BMP and Wnt pathways as inhibition of the BMP and Wnt pathways through either the BMP inhibitor Noggin or a Wnt inhibition dominant negative Xdd1 respectively reduced the signal from a YAP-TEAD-GFP reporter as compared to control (Kumar, Nitzan, and Kalcheim 2019). In addition, the transcription factor PAX3 is essential for neural crest survival and migration (M. Xu et al. 2018; Lang et al. 2005; Terzić and Saraga-Babić 1999; Lang et al. 2000; Kubic et al. 2008). YAP is co-expressed with PAX3 in the pre-migratory and migratory neural crest, and YAP promotes

PAX3 expression through a TEAD binding element within a PAX3 gene regulatory enhancer (Gee et al. 2011; Manderfield et al. 2014). Other than PAX3, the interaction of YAP with TEAD factors in the neural crest drives a number of other regulatory pathways within the neural crest. For example, YAP and TEAD1 complexes bind to a number enhancers upstream of genes that promote migration (such as ZEB1 and SNAI1), and the YAP/TEAD1 complexes are found on the enhancers prior to migration (Bhattacharya et al. 2020; Kumar et al. 2019). Another cellular cue that promotes neural crest migration is through the induction of glycolytic flux. A YAP-TEAD-GFP reporter construct consisting of multiple TEAD binding sites upstream of a minimal promoter driving GFP transfected into avian neural tube revealed that YAP-TEAD activity was repressed in the presence of 2-deoxy-D-glucose (2-DG), an inhibitor of glycolysis. This suggests that YAP and TEAD1 interaction is enhanced by glycolytic flux, which occurs concurrently with the induction of migration (Bhattacharya et al. 2020). While neural crest cells and melanoma may have different functions, neural crest derived tumors including melanoma activate many of the same molecular pathways that were used during neural crest development prior to metastasis during carcinogenesis and subsequently utilize many of the same pathways that drove the migration process during development (Tsoi et al. 2018). Indeed, it is telling that injection of SK-Mel28 melanoma cells into the avian neural tube resulted in the tumor cells following trunk migratory pathways and no tumor formation, whereas B16 melanoma cells injected into non-neural crest environments of the avian embryo resulted in melanoma formation (Menzel et al. 2014a; Schriek et al. 2005; Oppitz et al. 2007). A more in depth understanding of the role of YAP and TAZ plays in the neural crest may have direct application to melanoma biology.

YAP and TAZ are also both expressed in normal melanocytes and cultured melanocyte cells (Kim et al. 2013; Zhang et al. 2019). The expression of both factors is low in normal mature resting melanocytes, but is elevated in benign nevi and in melanoma cells (X. Zhang et al. 2019). There is support that increased levels of YAP promote a melanocyte lineage over other neural crest progenitor types. Increased YAP levels negatively correlate with a neural cell differentiation of neural crest cells, and ectopically overexpressed YAP promotes a melanocyte lineage over others, such as sensory neural cells (Hindley et al. 2016; Kumar, Nitzan, and Kalcheim 2019). Furthermore, conditions that support high levels of aerobic metabolism further promote the melanocyte phenotype over other neural crest progenitors (Bhattacharya, Azambuja, and Simoes-Costa 2020). One factor crucial for lineage specificity of melanocytes, as well as the proper development, maturation, proliferation, and survival of melanoblasts (a melanocyte precursor) is the gene MITF (microphthalmia transcription factor). With regards to melanocytes, MITF is often known as the "master regulator". MITF is essential for melanocyte lineage development, where it drives expression of the pigment producing genes DCT, TYRP1, and tyrosinase (Cichorek et al. 2013; McGill et al. 2002; Lang et al. 2005; Cooper and Raible 2009). YAP and TAZ directly promote MITF expression in PAX3-dependent mechanism (Manderfield et al. 2014; Miskolczi et al. 2018). Through MITF, YAP and TAZ promotes both melanocyte lineage and cellular proliferation. Although the knowledge of YAP and TAZ function in mature melanocytes is limited, as melanoma cells originate from melanocytes, many genetic pathways found in melanocytes and melanoblast development are most likely similar to those studied in melanoma.

1.5 Genetic drivers of melanoma and their potential therapeutic impact

As described earlier, cutaneous melanoma originates from melanocytes, pigment producing cells located in the basal layer of the epidermis. More importantly, cutaneous melanoma has a rising incidence rate and a high rate of metastasis. Aside from YAP and TAZ, other factors and genes have been more comprehensively studied in melanoma to date. For example, there are inherited genetic mutations that lead to susceptibility to melanoma. Approximately five to ten percent of cutaneous melanoma occurs in families (Gandini et al. 2005; Rossi et al. 2019). The most common familial mutations occur within the CDKN2A gene locus, which encodes the p16INK4A and p14ARF cell cycle regulatory proteins. The inherited mutations solely affect the p16INK4A gene, and not p14ARF, with more than 60 germline mutations identified (Hussussian et al. 1994; Kamb et al. 1994). These mutations tend to result in abrogation of functional p16INK4A and p14ARF, resulting in uncontrolled cell division. The second most common mutation identified to date is found in the CDK4 (Cyclin Dependent Kinase 4) gene. This familial mutation is rare, with all known mutations within exon 2 that encodes the p16 binding epitope (Zuo et al. 1996). These mutations are thought to make the protein more active, resulting in uncontrolled cell proliferation in the melanocytes. Both the familial mutations in CDKN2A and CDK4 result in high penetrance for the development of melanocyte disorders including melanoma. Other gene mutations identified that lead to high propensity of melanocytic dysfunction include the deubiquitinating enzyme BAP1 (breast cancer associated protein 1) and the telomere length regulating POT1 (protection of Telemeres 1) (Wiesner et al. 2011; Robles-Espinoza et al. 2014; J. Shi et al. 2014). BAP1 mutations are very well characterized but are thought to result in non-functional BAP1 protein and an increase in

cell proliferation. POT1 helps to regulate telomere length and stability and mutations to this gene result in chromosome instability. Additionally, rare mutations are found in the TERT (Telemerase Reverse Transcriptase) gene, although these mutations are found in the enhancer regions of the gene rather than the coding sequences (Horn et al. 2013; Harland et al. 2016). This results in lower expression of functional telomerase which results in chromosome instability and a higher susceptibility to melanoma. While mutations in BAP1, POT1, or TERT have been identified, they are very rare occurrences. Other familial mutations have been identified, but are not considered high penetrance for the development of melanoma. Rather, these genetic changes leave individuals prone to a higher risk for melanoma. The most common are alterations in the MC1R gene, which encodes a G-protein coupled receptor for alpha melanocyte-stimulating hormone (aMSH). Polymorphisms for the MC1R gene are common in Caucasian populations and leads to a reduction of black melanin and blond or red hair color. This reduction in melanin results in greater DNA damage from UV irradiation for people with this mutation. MC1R variants are associated with fair skin, freckles, sun sensitivity, a poor ability to tan, and a higher risk of melanoma (Palmer et al. 2000; Kennedy et al. 2001). Furthermore, rare mutations in MITF lead to a melanoma risk. Due to the role of MITF in melanocyte development, a genetic link to melanoma susceptibility is not surprising. The rare mutation, MITF E318K, alters an epitope that normally can be sumoylated, and the genetic alteration enhances the transcriptional function of MITF, resulting in increased cell growth and dysregulation of melanocyte function (Yokoyama et al. 2011; Bertolotto et al. 2011). Most of the mutations found in melanoma are non-familial, spontaneous mutations. The most common are in the genes B-RAF (50-60%) or N-RAS (15-20% of melanoma patients) and are for the most part mutually exclusive; melanomas

rarely have mutations in both of these genes. These mutations by themselves cannot induce melanoma, and may be early initiating mutations since they are also found in the form of benign melanocytic lesions (Vredeveld et al. 2012). B-RAF or N-RAS mutations result in constitutively active proteins which enhance proliferation and often induce cellular senescence within these benign nevi. For the formation of melanomas, overcoming cellular senescence is a crucial step that is often accomplished through abrogation of suppressor genes PTEN, P53, or P16 (Crowson et al. 2007; Ha, Merlino, and Sviaerskaya 2008). With regards to YAP and TAZ, mutations in YAP have been identified, where Serine epitopes that are known as phosphorylation targets from HIPPO pathway kinases are altered to phosphorylation resistant alanine residues (Zhang et al. 2019). However, this is a rare mutation and the majority of melanomas overexpress YAP and TAZ from genes without any obvious mutations (Bhattachary et al, 2020).

Genetic mutations, overexpressed genes, and the immune response are all potential targets for next generation melanoma therapeutics. The development of the small compound (PLX4032/RG7204) Vemurafenib targeting a V600E mutation (found in ~50% of all melanoma patients) in B-RAF kinase has produced an astounding 80% response rate for patients with that specific B-RAF mutation (Yang et al. 2010). A caveat to this miraculous compound is that it is only effective in melanoma patients with the V600E B-Raf mutations and those that receive this therapeutic drug develop eventual drug resistance followed by rapid progression of melanoma (Yang et al. 2010; Comin-Anduix et al. 2010; Niehr et al. 2011). A second therapeutic avenue that has shown great promise in treating melanoma and positively affecting patient outcomes is through blockage of the immune checkpoint receptor PD-1 with antibody treatment (Robert, Long, et al. 2015; Robert, Karaszewska, et al. 2015). One method T-cells use to distinguish

normal cells from tumor cells is through binding of their PD-1 receptor found on the cell surface of T-cells with PD-L1 antigen on the surface of normal cells. Melanoma cells can often escape this immune response by upregulation of PD-L1. PD-1 antibody treatment abrogates this ability of melanoma cells to avoid the immune response. PD-L1 Treatment with the PD-1 antibody Nivolumab led to a 1 year survival rate of 72.9% vs 42.1% in the group treated with traditional chemotherapeutic agent dacarbazine (Robert, Long, et al. 2015). Supplementing these two therapies, combination treatments with other known small molecule inhibitors have provided new therapeutic avenues (Robert, Karaszewska, et al. 2015; Ascierto et al. 2019). A potential caveat to these efficacious treatments is that many of these drugs have adverse side effects for the patient or the tumors develop resistance. As such, it is essential to study potential drivers of melanoma progression and survival to provide additional molecular targets for melanoma therapy.

YAP and TAZ have potential as targets for therapy, since these factors are overexpressed in human melanoma and their higher expression correlates with lower patient survival (Menzel et al. 2014b). While there are limited studies in how YAP and TAZ drive melanoma, there is evidence that suggest YAP and/or TAZ play crucial roles in melanoma progression (Kumar, Nitzan, and Kalcheim 2019; M. H. Kim et al. 2016; Lamar et al. 2012; Nallet-Staub et al. 2014). Specifically, in addition to reducing the ability of melanoma cells to invade into matrigel, YAP/TAZ knockdown in 1205Lu melanoma cells impaired the number of lung metastasis following tail vein injections in immunodeficient mice (Nallet-Staub et al. 2014). Furthermore, overexpression of YAP induced resistance to Vemerafenib and upregulated expression of the checkpoint inhibitor ligand PD-L1 to inhibit the immune response (M. H. Kim et al. 2016).

While these early studies suggest a role for YAP and TAZ in driving melanoma metastasis and proliferation, several of the studies do not directly investigate either YAP or TAZ alone. It is possible that one of the transcriptional coactivators play unique roles in melanoma and a better understanding of the potential differences between YAP and TAZ will provide a greater foundation for driving next generation melanoma therapeutics.

1.6 Concluding remarks and future challenges

In the past 2 decades, studies into YAP and TAZ have shown a remarkable ability to drive a wide variety of cellular mechanisms, including but not limited to development, regeneration, stem cell maintenance, tumor cell proliferation, and metastasis. Just as the downstream activity of YAP and TAZ is extremely diverse, regulation of YAP and TAZ have been shown to be just as varied, coming from a variety of extracellular and intracellular cues. In this chapter, we provided an overview of YAP and TAZ regulation and protein structure, while focusing on differences between the two. Furthermore, we highlight how these differences could be driving tumorigenesis and how this could affect potential therapeutics.

While there is a strong foundation in place for YAP and TAZ biology and its role to cancer development and progression, several key questions remain. As we have partially touched upon in this chapter, YAP and TAZ biology is very context and cell type specific. A full exploration and understanding of the key differences between the domains found in YAP and TAZ, namely the extra WW domain, the SH3 binding, and proline rich region found in YAP as well as the unique CC domain and N-terminal phosphodegron in TAZ, will be essential in elucidating how these two transcriptional cofactors are being coerced into driving various

cancers, including melanoma. Elucidation of YAP and TAZ specific pathways will provide a foundation to better understand the mechanisms by which different domains drive YAP and TAZ function in specific cancers. In turn, this will provide a better foundation to develop novel melanoma YAP, TAZ, or YAP/TAZ therapeutics.

In this dissertation, I focus on YAP and TAZ regulation of melanoma. In chapter 2, I describe functional and transcriptional differences between YAP and TAZ in melanoma cell lines. Additionally, I describe a YAP potential mechanism in driving melanoma migration through regulation of ARPC5. In Chapter 3, I demonstrate that a small molecule inhibitor of YAP and TAZ did not reduce melanoma progression or initiation in a transgenic melanoma mouse model. Taken together, these studies demonstrate that YAP drives melanoma progression in a unique and nonredundant fashion from TAZ and provide a rationale for future research towards the development of YAP, but not TAZ, targeted therapies in melanoma.

CHAPTER 2: YAP PLAYS A PREDOMINANT ROLE IN DRIVING MELANOMA MIGRATION

2.1 ABSTRACT

Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ-Binding Motif (TAZ) are transcriptional coactivators that have been implicated in driving metastasis and progression in many cancers, mainly through their transcriptional regulation of downstream targets. Although YAP and TAZ have shown redundancy in many contexts, it is still unknown whether or not this is true in melanoma. Here we show that while both YAP and TAZ are expressed in a panel of melanoma cell lines, depletion of YAP results in decreased cell numbers, focal adhesions, and the ability to invade matrigel. Using non-biased RNA-sequencing analysis, we find that melanoma cells depleted of YAP, TAZ, or YAP/TAZ exhibit drastically different transcriptomes. We further uncover the ARP2/3 subunit ARPC5 as a specific target of YAP but not TAZ. Our findings suggest that in melanoma, YAP drives melanoma progression, survival, and invasion.

2.2 AUTHOR CONTRIBUTIONS

The majority of the work presented here was performed by myself. However, several individuals worked alongside me to conduct several of the experiments. Kelsey Ogomori helped to conduct the RT-qPCR experiments (**Figure 2.5**). Lee Huang helped in performing the PLA experiments (**Figure 2.7**). Yan Li and Stephen Moore helped drive the RNA-seq analysis. This chapter is currently under revision as manuscript coauthored with Kelsey Ogomori, Lee Huang, Stephen Moore, Yan Li, and Deborah Lang.

2.3 INTRODUCTION

Melanoma is an aggressive cancer with a high degree of metastasis (Tas 2012). Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ-Binding Motif (WWTR1 or TAZ) are transcriptional coactivators implicated in driving metastasis and progression in a variety of cancers, including melanoma (Zanconato, Cordenonsi, and Piccolo 2016; Zhao et al. 2008; Nallet-Staub et al. 2014; Lamar et al. 2012). In melanoma, increased YAP and TAZ expression correlates with lower patient survival (Guo, Kang, and Zhao 2018; Menzel et al. 2014b). Furthermore, loss of YAP and TAZ led to decreased invasion and tumorigenicity in 1205 Lu and SKMEL-28 melanoma cell lines (Nallet-Staub et al. 2014). In other cancer types, both YAP and TAZ have been implicated in driving various aspects of metastasis. In pancreatic cancer, YAP overexpression drives metastasis by activating the AKT cascade to induce the epithelial to mesenchymal transition (Yuan et al. 2016). In hepatocarcinoma cells, reduction of YAP phosphorylation via LATS2 inhibition reduced and increased EMT markers E-cadherin and vimentin respectively (L. L. Han, Yin, and Zhang 2018). In colorectal cancer, YAP is shown to promote the epithelial to mesenchymal transition by driving expression of MALAT1, which in turn promotes expression of various metastasis markers, including VEGFA, SLUG, and TWIST. In addition, TAZ upregulation is able to rescue migratory and invasive phenotypes exhibited with miR-125a-5p inhibition (L. Tang et al. 2019). Outside of metastasis, YAP and TAZ have been shown to drive several other cancer cell processes, including cell proliferation, differentiation, and drug resistance (Fisher et al. 2017; Zhao et al. 2008b; Zanconato et al. 2015; Choe et al. 2018).

YAP and TAZ contain many similarities and potential redundancies. Structurally, YAP and TAZ share 60% protein sequence (Plouffe et al. 2018; W. Hong and Guan 2012). They do not contain a DNA binding domain, so both YAP and TAZ require binding partners that contain DNA binding domains to exert their downstream effects. Traditionally, both YAP and TAZ bind to the TEAD family of transcription factors, but either YAP, TAZ, or both have been shown to bind to the RUNX, SMAD, and hnRNP family of proteins, among others (Zanconato, Cordenonsi, and Piccolo 2016; Yagi et al. 1999; Kanai et al. 2000; J. H. Hong et al. 2005; Ferrigno et al. 2002; Howell, Borchers, and Milgram 2004). Transcriptionally, both YAP and TAZ have been shown to directly control expression of CTGF and Cyr61, genes implicated in cell differentiation and cell adhesion, among others (Haiying Zhang, Pasolli, and Fuchs 2011; Zhao et al. 2008). Due to past studies on *Yorkie* in *D. melanogaster* and YAP/TAZ in *H. sapiens*, it is clear that YAP and TAZ have overlapping activity in driving diverse biological functions.

While it is clear that YAP and TAZ share many functions, there is also evidence that these factors are not just redundant paralogs. Although YAP and TAZ contain many of the same protein binding domains, both proteins also contain unique binding domains of their own, most notably YAP containing an extra WW domain, an SH3 binding motif, and an N terminal proline rich region (Varelas 2014). Phenotypically, several studies show stark differences in functionality between the two transcriptional cofactors. YAP knockout mice are embryonic lethal, whereas TAZ mice can live until adulthood but exhibit both kidney and lung defects (Morin-Kensicki et al. 2006; Hossain et al. 2007; Makita et al. 2008; Tian et al. 2007). Lastly, when differentially expressed genes from HEK293 YAP KO and TAZ KO cells were compared to YAP/TAZ KO cells, only 81% and 41% of the differentially expressed genes overlapped with

the YAP/TAZ KO group respectively (Plouffe et al. 2018). While these studies, among others, reveal many overlapping roles for YAP and TAZ in a variety of biological settings, it is still unknown what unique functions YAP and TAZ may also be performing.

In this study, we have identified YAP as the predominant player in our melanoma cell lines. We show that YAP specific inhibition leads to a reduction in melanoma cell numbers, invasion, and focal adhesions. Furthermore, RNA-sequencing reveals a YAP transcriptome highly distinct from TAZ and more enriched in genes involved in cancer progression. We demonstrate direct inhibition of one such YAP target gene, ARPC5, leads to a decrease in focal adhesion numbers, melanoma cell migration, and a shift in the ARP2/3 complex subunits. Taken together, our data support a model whereby YAP drives ARPC5 expression to enhance melanoma cell migration.

2.4 MATERIALS AND METHODS

Cell Culture

Human melanoma cell lines A375, M14, mel537, mel624, mel888, SKMEL-28, SKMEL-23, SKMEL-5, and mouse melanoma B16 cells (ATCC, Manassas, VA and University of Chicago Comprehensive Cancer Center Core Facilities) were cultured in DMEM with 10% FBS (Sigma-Aldrich). Melanoma marker testing, morphology, and histological analysis were used to verify melanoma cell identity and lack of mycoplasma contamination.

Immunoblotting

Thirty μg total protein melanoma lysates (MPER, Thermo Fisher – 78501) were separated on 4-15% Bis-Tris gels (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were incubated overnight with antibody (Dilutions - 1:1000 for YAP/TAZ antibody (Cell Signaling Technology – D24E4), 1:1000 ARPC5 (Proteintech - 16717-1-AP), 1:1000 ARPC5L (Proteintech – 22025-1-AP), 1:10000 for GAPDH (Cell Signaling – D16H11)) in nonfat 5% milk (Santa Cruz – sc-2324). The membranes were washed with 1X TBS-T four times for 15 minutes and incubated with 1:4000 anti-rabbit IgG HRP-linked antibody (Cell Signaling – 7074). Membranes were developed with Clarity Western ECL substrate according to the manufacturer's instructions (Bio-Rad). Blots were normalized with GAPDH or total protein input (Stain Free System, Bio-Rad). All Western analysis shown are representatives of at least three independent experiments.

Densitometry

Western blot band intensities were measured using ImageJ64 (https://imagej.nih.gov/ij/download.html). All experiments were performed in triplicate, with each figure showing a representative image. The data shown are represented as a percentage of siScrambled controls and normalized to total protein.

siRNA treatments

Melanoma cells were seeded at 50-70% confluency in 6-well plates. siRNA transfection

using Lipfectamine 3000 (Thermo Fisher) were then performed according to the manufacturer's instructions with siRNA targeting YAP, WWTR1 (TAZ), ARPC5, and/or siScrambled (Thermo Fisher ID Number S20366 – YAP1, S24787 – WWTR1, S19362 – ARPC5, 4390844 – siScramble). Cell lysates were collected 2 days post-siRNA transfection.

Cell shape morphology

Cell pictures were taken with a Q-Color3 Olympus camera on an Olympus CKX41 microscope 2 days post transfection. Cell shape was quantified by fitting the cell shape to an ellipse and calculating the ratio between the short and long axis utilizing photoshop measurement tool (PS Version CS6, Adobe Inc). 200 cells were counted per experiment, performed in triplicate.

Growth Curves Analysis

Cell confluency was measured every 6 hours over a period of 72 hours using the Incucyte FLR Live-Cell Imaging System (Essen BioScience, 2011A software). All experiments were performed in triplicate and all experimental groups were normalized to siScrambled control groups at 72 hours post treatment set at 100%.

Invasion assays

All matrigel invasion assays were performed using the Corning Matrigel matrix (Cat. No. 356234) on 8.0 μm pore cell culture inserts (Fisher) according to the manufacturer's instructions with 10% FBS in DMEM as the chemoattractant (0.5% FBS in DMEM as the base media) and

visualized using the Diff-Quik staining kit (Thermo Fisher). All analysis was performed by comparing the number of stained cells in the experimental groups as fold change compared to siScrambled control. All assays shown are representatives of at least three independent experiments, where cells were counted in 4 separate random fields of the cell culture insert.

Migration assays

Melanoma cells were plated to 90-100% confluency (50,000 cells per well). Scratch wounds were made using Essen BioScience's 96 well woundmaker according to manufacturer's instructions. Pictures were taken immediately post wound and 24 hours post wound using the Incucyte FLR Live-Cell Imaging System (Essen BioScience, 2011A software). Cell migration was analyzed by calculating the percentage of the distance of the wound at time zero and 24 hours post wound. All experiments were performed in triplicate and all experimental groups were normalized to siScrambled control groups set at 100%.

Immunofluorescence

Melanoma cells grown on coverslips were washed 2x in prewarmed PBS, fixed in 4% paraformaldehyde at 37 °C, washed 2x in prewarmed PBS, and then permeabilized for 15 minutes in PBS with 0.1% Triton X-100. Blocking was performed for an hour using 5% horse serum in PBS-T, followed by incubation in anti-vinculin antibody (Millipore Sigma – 05-386) diluted 1:5000 in 5% horse serum in PBS-T overnight, washed 2x PBS-T, and incubated in DyLight horse anti-mouse secondary antibody (Vector Laboratories – DI-2488) diluted 1:200 in 5% horse serum for 2 hours. Coverslips were washed 2x with PBS-T and stained for actin using Alexa Fluor 555 Phalloidin (ThermoFisher – A34055) according to the manufacturer's

instructions. Images were captured using a Nikon Eclipse E400 microscope. Puncta were quantified by counting the number of puncta where the actin filament met the vinculin puncta. All experiments were performed in triplicate and all experimental groups were normalized to siScrambled control groups set at 100%.

RNA Sequencing

All RNA sequencing experiments were performed on mel537 cells in duplicate from each group (siScrambled, siYAP, siTAZ, siYAP/TAZ). RNA was collected using Direct-zol RNA Miniprep Kit (Zymo Research – R2060) and submitted to the University of Chicago Genomics Facility (http://fgf.uchicago.edu) for library preparation and RNA sequencing on the Illumina HiSeq4000 platform (single-end 50 basepair). Subsequent bioinformatics analysis was performed by the Center for Research Informatics (http://cri.uchicago.edu) and Gene Ontology analysis was performed through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Gene set enrichment analysis was performed through use of the RaNA-seq tool (https://ranaseq.eu/home, GO:0005925,(Prieto and Barrios 2019)).The false discovery rate was set at $p<0.05$ and the cutoff for differentially expressed genes was a fold change of >1.5 as compared to siScrambled control.

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from melanoma cells using the Direct-Zol RNA extraction kit instructions (Zymo Research - R2071, R2050-1-200) according to the manufacturer's instructions. cDNA was then generated using the iScript cDNA Synthesis kit (Bio-Rad). The expression levels of YAP, TAZ, and ARPC5 were analyzed using SYBR-Green Master Mix

(Bio-Rad) in conjunction with the CFX Connect Real-Time System (Bio-Rad), and normalized to GAPDH. The primers used were as follows (5'->3'): YAP1F - GTG AGC CCA CAG GAG TTA GC; YAP1R - CTC GAG AGT GAT AGG TGC CA; YAP2F - TCT TCC TGA TGG ATG GGA AC; YAP2R - GGC TGT TTC ACT GGA GCA CT; TAZF - GTA TCC CAG CCA AAT CTC G; TAZR - TTC TGA GTG GGG TGG TTC; GAPDHF - ACA TCA TCC CTG CCT GTA CT; GAPDHR - CTC TCT TCC TCT TGT GCT CTT G; ARPC5F - AGA GCC CGT CTG ACA ATA G; ARPC5R - CAG TCA AGA CAC GAA CAA TG

Meta Analysis

RNAi (DEMETER2) and CRISPR (CERES) dependency scores were obtained from the DepMap website (https://depmap.org/portal/, (Z. Tang et al. 2017)). Protein expression scores for normal tissue vs cancer were obtained from the web server GEPIA (http://gepia.cancer-pku.cn/index.html). Expression scores for cutaneous melanoma vs benign nevi were obtained from Oncomine (https://www.oncomine.org). Oncoprints for members of the ARP2/3 complex were obtained from cBioPortal (cbioportal.org).

Proximity ligation assay

Melanoma cells were treated as described above, and then transferred to 24 hours posttreatment to 6 well plates containing flame sterilized cover slips, where they were to attach for 24 hours. The proximity ligation assay was then performed according to the manufacturer's instructions (Sigma-Aldrich - DUO 92101) using antibodies for ARP2 (Santa Cruz – sc 166103) and ARPC5L (Proteintech – 22025-1-AP).

Statistical analysis

Statistics were performed using student's two tailed T-test between control and the appropriate groups. All experiments were performed in triplicate, with a representative experiment shown in the figure, and all findings stated as significant have $p < 0.05$ unless otherwise stated.

2.5 RESULTS

2.5.1 YAP specific inhibition results in a decrease in melanoma cell number, invasive capacity, and morphological changes

Recently, the Broad institute generated predictive modeling software through the compilation of large-scale genomic screens from RNAi, CRISPR, and drug target experiments in a variety of cancer cell lines to identify potential targets that the cancers may be dependent on. Using the Cancer Dependency Map, we initially sought to determine whether or not melanoma demonstrated a specific dependency on YAP and TAZ. YAP (**Figure 2.1 A, C**), but not TAZ (**Figure 2.1 B, D**) trended with melanoma (**Figure 2.1 – Groups 1,4**) in both CRISPR (**Figure 2.1 A, B**) and RNAi (**Figure 2.1 C, D**) screens. While blood and solid tumors are both dependent on YAP and TAZ in the CRISPR and RNAi screens, melanoma and skin tumors are only YAP dependent. Based on these results from an unbiased large-scale genetic screen, melanoma exhibits different dependencies with regards to YAP and TAZ biology and function.

To determine expression levels of both YAP and TAZ in melanoma cells, Western blot analysis was performed on eight melanoma cell lines (1 mouse, 7 human) for the presence of YAP and TAZ proteins. Utilizing a dual YAP/TAZ antibody, all 8 cell lines examined expressed

varying degrees of YAP and TAZ, with 6/8 cell lines showing higher expression of TAZ than YAP (**Figure 2.2A**). Mel537 cells showed relatively equal expression of YAP and TAZ, while SKMEL-23 cells showed higher YAP expression than TAZ (**Figure 2.2A**). Higher exposure of the blots detect TAZ expression in SKMEL-23 cells, but the other cell lines are overexposed (data not shown). To test the effects of YAP and/or TAZ inhibition in our cells, we first needed to verifYAP and TAZ specific siRNAs in our melanoma lines (**Figure 2.2B**). Initial observation of the various knockdown groups (siYAP, siTAZ, siYAP/TAZ) as compared to siScrambled control revealed dynamic morphological changes in the siYAP and siYAP/TAZ, but not siTAZ, knockdown groups, resulting in more elongated cells as compared to siScrambled control (**Figure 2.2C**). As compared to the siScrambled groups which showed a length to width ratio of 1.99±1.25, 1.73±0.97, and 2.54±1.33 for mel537, A375, and m14 cell lines, siYAP and siYAP/TAZ knockdown groups had ratios of 3.51±2.58/2.92±2.13, 2.32±1.18/2.27±1.24, and 2.98±1.67/3.07±1.87 respectively. This was interesting, as the elongated phenotype more closely resembled differentiated melanocytes than melanoma cells. Cell growth is also YAP dependent, with cells significantly reduced to 75% or less of control levels (**Figure 2.2D,** $p<0.05$). In 3/6 of the lines (mel537, M14, UACC62), cell numbers decreased after 72 hours in a YAP specific manner (only in the YAP, or YAP/TAZ knockowns) while TAZ knockdowns were similar in growth to siScrambled control. The remaining three lines (A375, SKMEL5, SKMEL23) showed decreased cell numbers with inhibition of both YAP and TAZ. Next, we examined whether YAP, TAZ, or YAP/TAZ inhibition had an effect on invasion through the use of Matrigel invasion assays. As we saw two distinct patterns of growth inhibition, we utilized a representative cell line that show varying degrees of YAP vs TAZ expression from each growth pattern group (A375

and mel537). Both A375 and mel537 cell lines showed decreased invasion capacity with inhibition of YAP or YAP/TAZ, but not TAZ alone ($p<0.05$) (**Figure 2.2E**). Taken together, our observations suggest that YAP plays a predominant role in the morphology, cell growth, and invasion of melanoma cell lines.

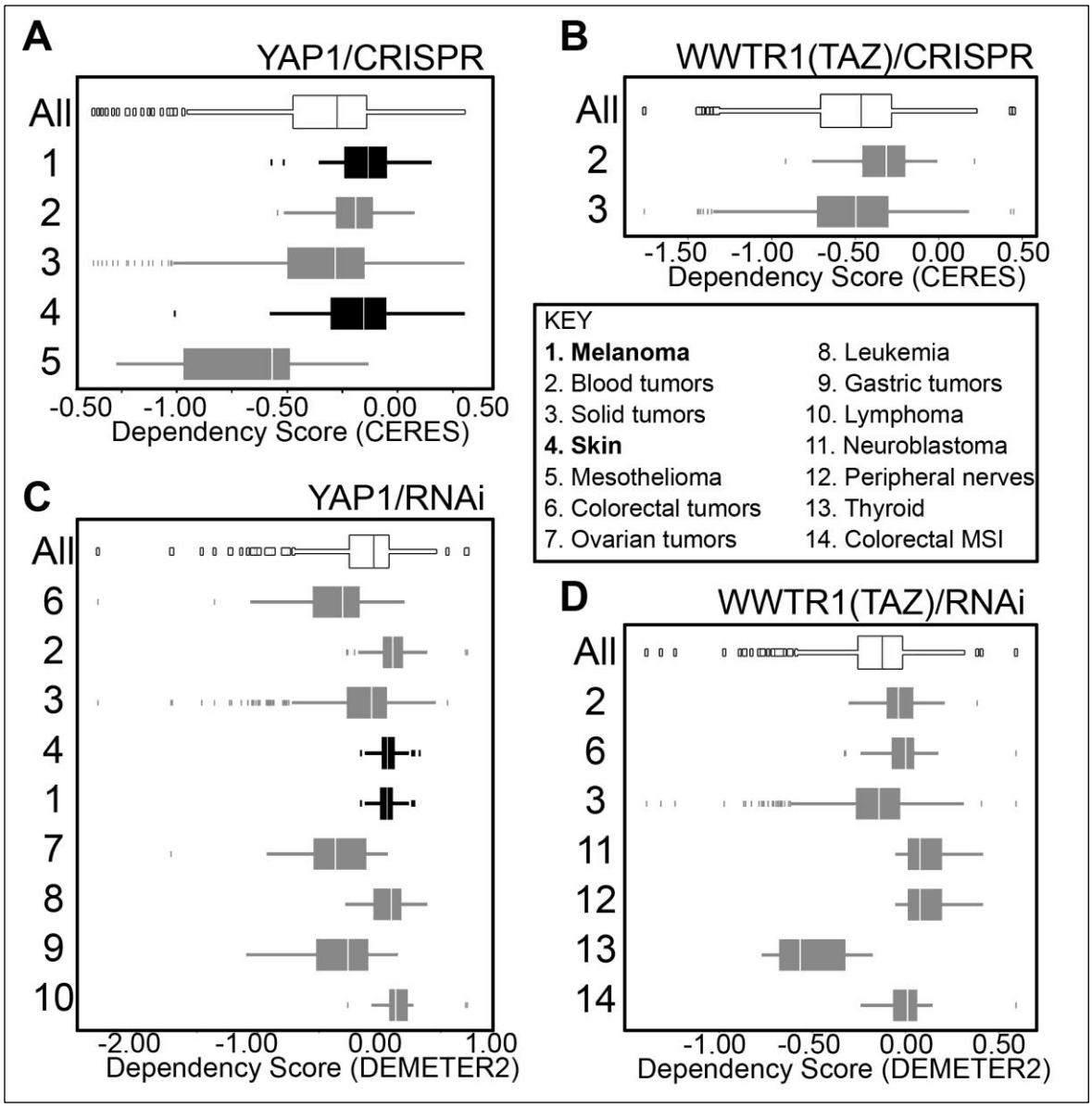

Figure 2.1: Melanoma is dependent on YAP, but not TAZ, in melanoma datasets. DepMap Portal analysis of YAP CRISPR (A), TAZ CRISPR (B), YAP RNAi (C), and TAZ RNAi (D).

Figure 2.1 (continued) CERES and DEMETER2 dependency scores corresponding to CRISPR and RNAi cell depletion assays. A lower score indicates higher probability of being essential in human cells. Black and grey bars represent melanoma related and all other cancer groups respectively. The white bars indicate the compilation of all cell lines and cancer types examined. All results shown are statistically significant (P<0.05).

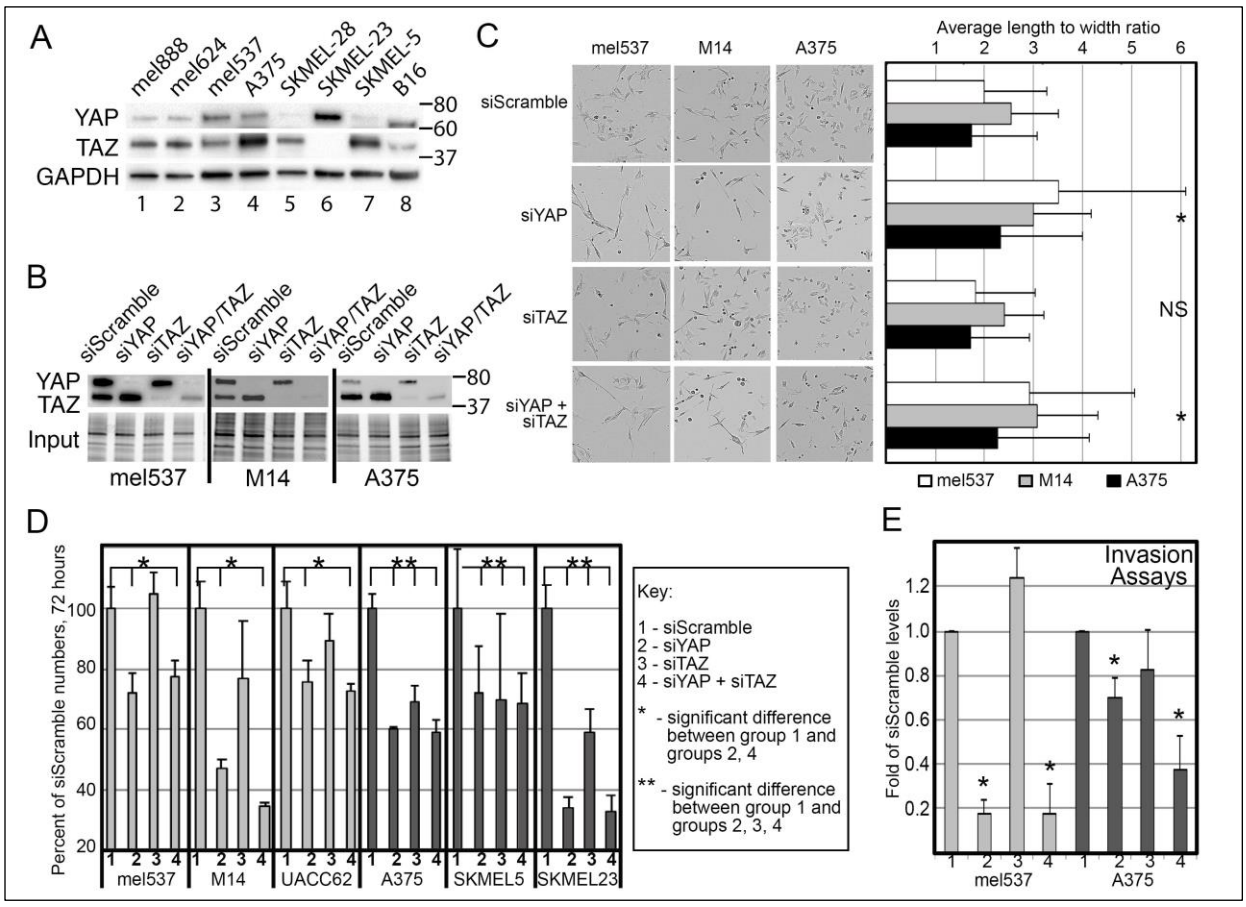

Figure 2.2. YAP inhibition results in decreased cell numbers and invasion in melanoma cells. (A) Melanoma cell lines express both YAP and TAZ at various levels. Western blot analysis for YAP and TAZ, with GAPDH as a loading control, was performed in a panel of melanoma cell lines (lanes 1-8). (B) Specific siRNA mediated knockdowns of YAP, TAZ, and YAP/TAZ. Western blot analysis measuring levels of YAP and TAZ were performed in mel537, M14, and A375 cells transfected with siYAP, siTAZ, or both siYAP and siTAZ. (C) Morphology of mel537, M14, and A375 melanoma cells change with inhibition of YAP, TAZ, or YAP/TAZ. Cells were best fit into an ellipse, where the cellular ratio of width to length was calculated, 48 hours post siRNA transfection targeting YAP, TAZ, both YAP/TAZ, or siScrambled control.

Figure 2.2 (continued) Representative photos of each cell line and knockdown group are shown. Values are mean ± SD (n = 200) (* indicates $p < 0.05$, NS indicates not significant). (D) Inhibition of YAP and TAZ result in decreased cell numbers. Cells were initially transfected with siRNA targeting YAP, TAZ, both YAP/TAZ, or siScrambled control and subsequently plated at 5-10% confluency. The confluency levels of the cells were then measured over 72 hours using the Essen BioScience IncuCyte Live-Cell imaging system. Images were taken every 6 hours for all knockdown groups and compared to siScrambled control. Values are mean ± SD. (*, $p < 0.05$). (E). Inhibition of YAP and TAZ result in decreased invasion. Matrigel invasion assays were performed on mel537 and A375 cells that were transfected with siRNA targeting YAP, TAZ, both YAP/TAZ, or siScrambled control. The values are expressed as the average of three independent experiments (*, $p < 0.05$).

2.5.2 Inhibition of YAP decreases the number of focal adhesions in melanoma cells

Since we saw several melanoma cell functions (morphology, cell numbers, invasion) specific for inhibition of YAP, but not TAZ, we predicted that inhibition of YAP may deregulate the ability of melanoma cells to migrate through regulation of focal adhesion numbers, as focal adhesion dynamics have been shown to be important for cell migration and cancer metastasis (Mitra and Schlaepfer 2006; Nagano et al. 2012). To visualize and quantify focal adhesions, we performed a series of immunofluorescent stains for vinculin (a key focal adhesion protein) and actin on mel537 (**Figure 2.3A**) and A375 (**Figure 2.3B**) cells treated with siRNA targeting YAP, TAZ, or both YAP/TAZ to examine changes in the number of focal adhesions as compared to siScrambled control. Quantification of the focal adhesion puncta (where actin and vinculin show colocalization) revealed that both cell lines demonstrated a significant loss in the number of focal adhesions per cell in a YAP specific manner ($p < 0.05$, n = 200 cells/group). YAP knockdowns had only 71±36% and 70±44% of vinculin puncta as compared to siScrambled in mel537 and A375 cells respectively. Similarly, YAP/TAZ knockdowns had 69±52% and 71±31% as compared to siScrambled group in mel537 and A375 cells. This indicates that the number of

focal adhesions is either directly or indirectly controlled by YAP in mel537 and A375 melanoma cells.

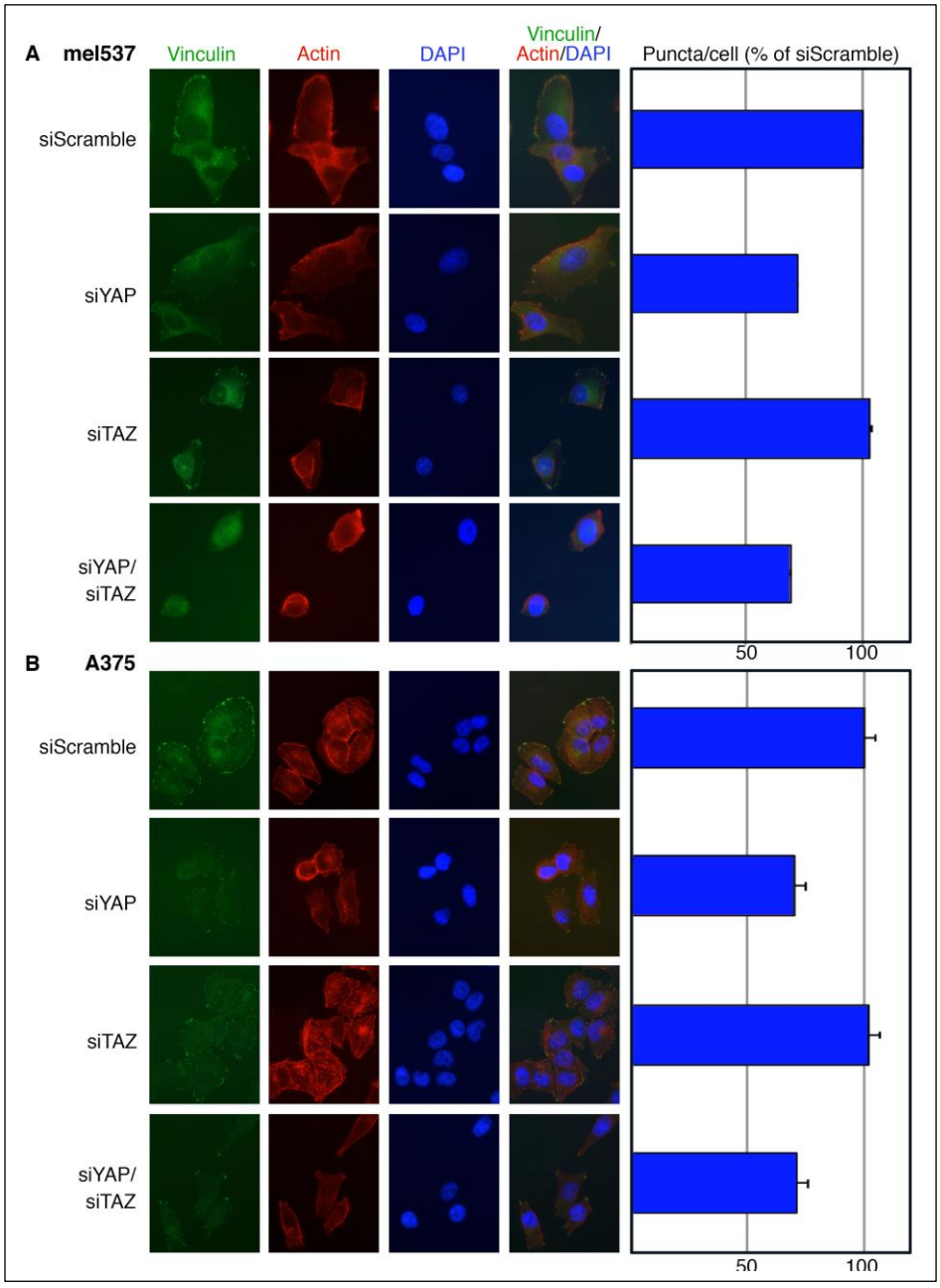

Figure 2.3: Inhibition of YAP decreases the number of focal adhesions in melanoma cells. Mel537 (A) and A375 (B) melanoma cells were transfected with siRNA targeting YAP, TAZ,

Figure 2.3 (continued) YAP/TAZ, or siScrambled control and transferred to coverslips, where they were allowed to adhere for 24 hours. The cells were fixed and stained with antibodies against vinculin (Alexa 488), phalloidin (Alexa 555) for actin, and DAPI for nuclei. The number of vinculin puncta, representing focal adhesions, were then counted for each cell (n = 50). Each experiment was performed in triplicate, and a single representative experiment is shown with photos of the stained cells on the left and graphs on the right with the average number of puncta per cell as a percent compared to puncta per cell of siScrambled control. Values are mean $\pm$ SD ($p < 0.05$).

2.5.3 YAP and TAZ have both overlapping and unique transcriptomes

Both YAP and TAZ are transcriptional co-regulators and exert their downstream effects through transcriptional regulation of target genes. Based on our initial results, we theorized that YAP plays a unique role from TAZ in melanoma and that the differences between YAP and TAZ physiology in our experiments were due to differences in downstream transcriptional regulation. As our earlier experiments supported the idea of YAP specific roles in melanoma, we performed RNA-sequencing analysis on YAP, TAZ, and YAP/TAZ inhibited mel537 cells to look for unique transcriptome signatures in our various knockdown groups. Mel537 cells were chosen as they exhibited the largest phenotypic changes with YAP knockdowns as compared to siScrambled control. To reduce false positives, we used a stringent criterion (FDR = $p<0.05$, LogFC>1.5) for analysis of our RNA-sequencing data. We grouped differentially expressed genes that were common between YAP and YAP/TAZ knockdowns (termed "YAP specific") and those between TAZ and YAP/TAZ knockdowns (termed "TAZ specific). There were 264 YAP specific and 96 TAZ specific differentially expressed genes (**Figure 2.4C**). Taking these two groups, we performed pathway analysis using Qiagen's Ingenuity Pathway Analysis software. Pathway analysis revealed stark differences in the molecular function and biological

processes between the two groups, with the YAP specific genes more highly enriched in cellular movement, growth, and development groups, whereas the majority of genes for the TAZ specific group were more oriented towards endocrine and inflammatory processes (**Figure 2.4A, 4B**). Several of the top differentially expressed genes for the YAP and TAZ specific groups are shown in **Figure 2.4D**. Taken together, YAP and TAZ have both overlapping and unique transcriptomes in melanoma.

2.5.4 ARPC5 drives a pro-migratory phenotype in melanoma cells

To look for potential YAP specific genes that controlled focal adhesion numbers, we performed gene set enrichment analysis (GSEA) for the siYAP and siTAZ knockdown groups. GSEA revealed focal adhesion enrichment for both of the groups but due to our previous results that demonstrated a loss of focal adhesion numbers only in our melanoma cells specific to YAP knockdowns, we hypothesized that genes enriched in the focal adhesion gene set for YAP but not for TAZ must be essential for the phenotype examined (**Figure 2.4E, 2.4F**). A closer examination of the differentially expressed genes for the YAP specific group revealed ARP2/3 complex member ARPC5 as a downregulated gene with YAP inhibition (**Figure 2.4D**). Inhibition of YAP alone led to a 3.25 fold decrease in ARPC5 transcript ($p = 5.4x10^{-19}$, FDR = $1.9x10^{15}$). As our earlier results showed decreased cellular migration, invasion, and a loss of focal adhesion numbers in YAP inhibited melanoma cells (**Figure 2.2, 2.3**), we speculated that ARPC5, as a member of the actin nucleating ARP2/3 complex, could modulate melanoma cell migration as a downstream target of YAP activity. To recapitulate our RNA-sequencing results, we examined ARPC5 protein and transcript expression in YAP, TAZ, and YAP/TAZ inhibited conditions in different cell lines. 5/5 melanoma cell lines examined exhibited reduced ARPC5

transcript under YAP, but not TAZ, inhibition (**Figure 2.5A**). To determine changes in protein levels, we performed western blots and subsequent densitometry analysis for ARPC5 expression

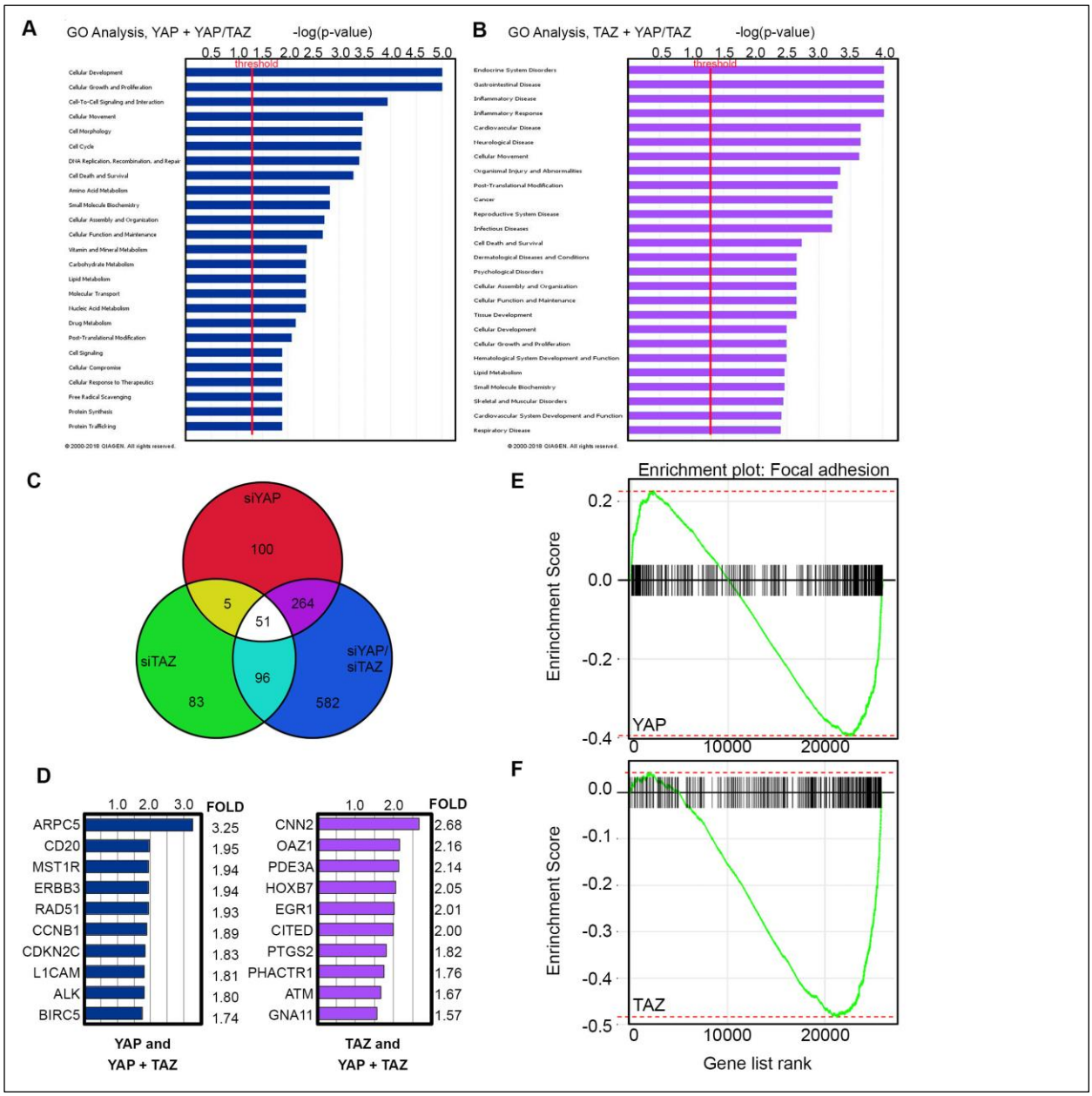

Figure 2.4: YAP and TAZ have both overlapping and unique transcriptomes. (A,B) Gene Ontology (GO) analysis of differentially expressed genes as detected by RNA-sequencing common to YAP and YAP/TAZ knockdowns (A), and to TAZ and YAP/TAZ knockdowns (B),

Figure 2.4 (continued) in mel537 melanoma cells. Data represented as –log(p-value), where significance is determined as being above the threshold as indicated by the red line. For the RNA-seq analysis, the false discovery rate was set at $p<0.05$ and a fold change of > 1.5 as compared to siScrambled control. (C) Venn Diagram schematic representing the total numbers of differentially expressed genes of the three groups (YAP knockdown, TAZ knockdown, YAP/TAZ knockdown. (D) Representative list of differentially expressed genes from the RNA-Sequencing. Data represented as the mean of the fold changes from the biological duplicates. (E-F) Gene Set Enrichment Analysis (GSEA) of siYAP (E) and siTAZ (F) RNA-sequencing samples (GO:0005925).

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in 3 melanoma cell lines under YAP, TAZ, and YAP/TAZ knockdown conditions. Inhibition of YAP expression led to decreased ARPC5 protein levels in mel537 (27.8±16.9% of controls, **Figure 2.5 B,C**), M14 (59.7±9.1%, **Figure 2.5 E,F**), and A375 (26.1±12.3%, **Figure 2.5 H, I**). Taken together, inhibition of YAP but not TAZ results in loss of ARPC5 expression at both protein and transcript levels in melanoma cell lines.

To determine if inhibition of ARPC5 will phenocopy YAP inhibition, we directly targeted ARPC5 in mel537 and A375 melanoma cells using siRNA specific for ARPC5. Inhibition of ARPC5 in mel537 and A375 cells did not produce significant changes in cell length, growth, or ability to invade into matrigel (**Figure 6A-C**). However, we did observe that both YAP and ARPC5 inhibition reduced migration in a wound healing assay for both mel537 and A375 cells. Compared to the siScrambled control groups, both YAP and ARPC5 knock down groups exhibited decreased migration (**Figure 6D**, $p<0.05$). For mel537 cells, direct YAP and ARPC5 knockdowns led to wounds that were only 73.6± 19.2% and 73±15.33% healed as compared to siScrambled control ($p<0.05$). For A375 cells, the percentages were 75.0±16.0% and 59.9±16.9% respectively ($p<0.05$). Furthermore, inhibition of ARPC5 also led to a reduction of focal adhesions in the cell lines examined. Similar to the YAP knockdowns, direct ARPC5

inhibition resulted in total numbers of puncta that were 70.5%±32.6 and 85.1±40% as compared to siScrambled controls for mel537 and A375 cells respectively (**Figure 2.6E**, $p<0.05$, n = 200 cells/group). To summarize, direct ARPC5 inhibition led to decreased numbers of focal adhesions and migration, but no differences in cell numbers or capacity to invade into matrigel (**Figure 2.6F**).

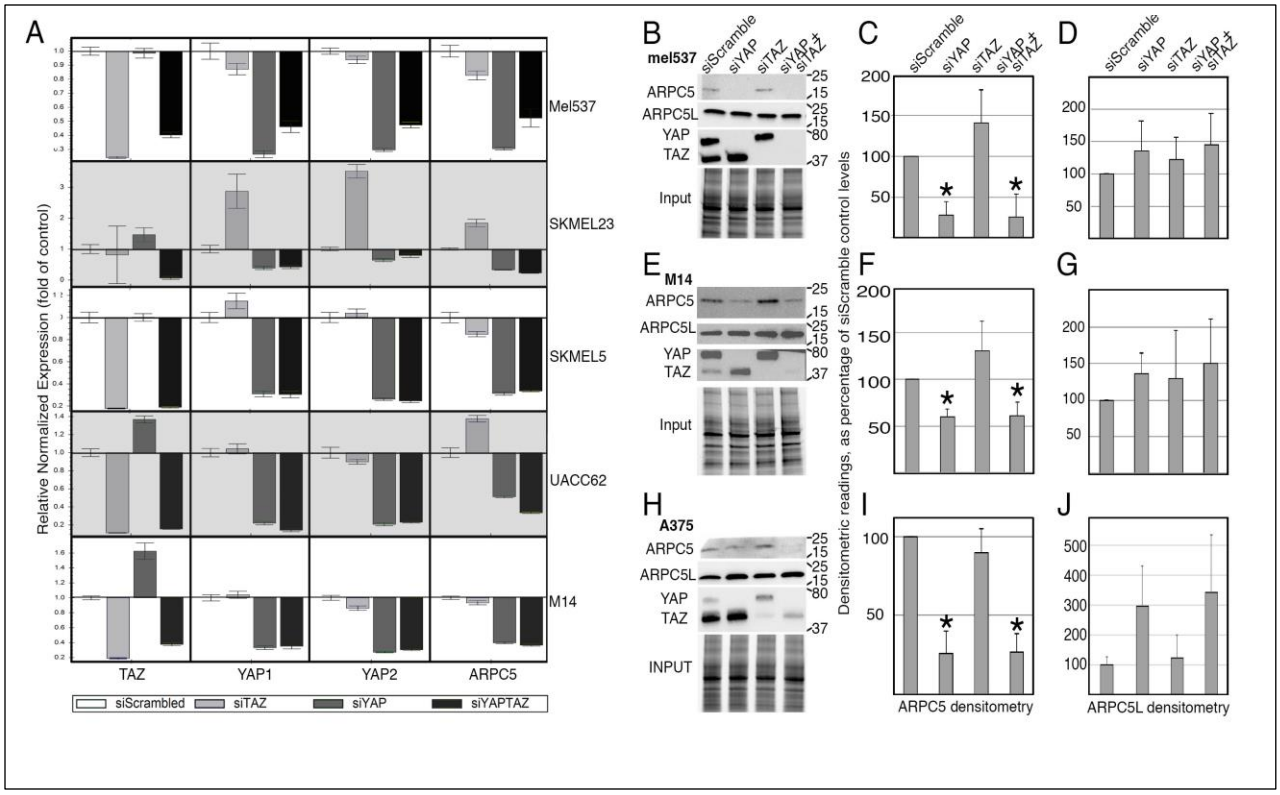

Figure 2.5: Inhibition of YAP results in a decrease in ARPC5 transcript and protein in melanoma cells. (A) Inhibition of YAP results in decrease of ARPC5 transcript in 5 Melanoma cell lines (mel537, SKMEL23, SKMEL5, UACC62, and M14). Quantitative Real Time Polymerase Chain Reaction analysis examined expression levels of ARPC5, YAP1, YAP2, and TAZ in melanoma cells treated with siRNA targeting YAP, TAZ, YAP/TAZ, or siScrambled control. The data presented are normalized to control expression levels (GAPDH). (B-J) Inhibition of YAP results in decrease of ARPC5 protein. Western blot analysis for YAP, TAZ, ARPC5, and ARPC5L for the knockdown groups (siScrambled, siYAP, siTAZ, siYAP/TAZ) for

Figure 2.5 (continued) the cell lines mel537 (B), M14 (E), and A375 (H). Densitometry quantification of the Western blot analysis for ARPC5 (C, F, I) and ARPC5L (D, G, J) are shown on the right for mel537, M14, and A375, respectively. All data represented as the mean of the fold changes from the biological triplicates are normalized to and represented as a percentage of siScrambled control. Values are mean ± SD. (*, $p < 0.05$).

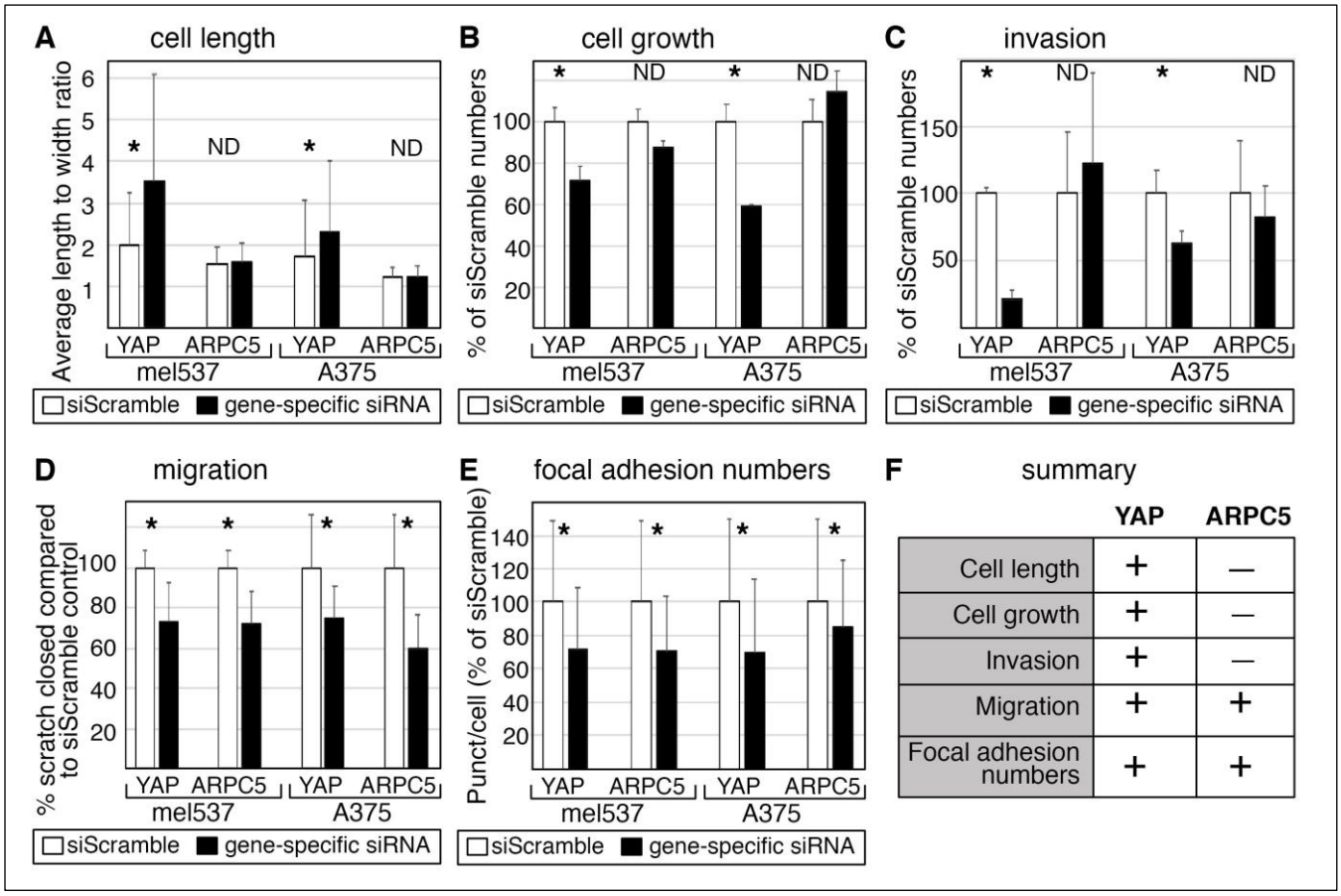

Figure 2.6: Direct inhibition of ARPC5 results in decrease of melanoma cell migration and focal adhesion numbers. (A,B,C) Direct ARPC5 inhibition does not phenocopy YAP inhibition in A375 and mel537 cells with cell morphology (A), growth (B), or invasion (C). Methods follow procedures outlined in Figure 2. (D,E) Direct ARPC5 inhibition phenocopies YAP inhibition for cell migration (D) and number of focal adhesions (E). For cell migration, the percent of wound

Figure 2.6 (continued) closure after 24 hours was calculated. For focal adhesion numbers, methods outlined in Figure 3 are followed. (F) Summary chart of phenotypes induced by direct ARPC5 or YAP inhibition. For A-E, white bars indicate siScrambled control and black bars indicate the gene specific siRNA knockdown group. Each experiment was performed in triplicate, and a single representative experiment is shown (*, $p < 0.05$) (ND = no significant difference)

2.5.5 ARPC5 and ARPC5L have an inverse relationship in melanoma

ARPC5 is a member of the 7 subunit ARP2/3 complex, a crucial regulator of actin nucleation, shown schematically in **Figure 7B** (Weaver et al., 2001). Meta-analysis of the melanoma TCGA dataset reveals that expression levels of each of the ARP2/3 family members are often altered in melanoma patients (percentage shown next to each subunit in **Figure 2.7A)**. Taken together, alterations to any of the ARP2/3 subunits occurs in 61% of melanoma patients from the TCGA dataset, and that the majority of these differences are due to increased expression, as opposed to mutations or gene duplications (**Figure 2.7A**).

In addition to ARPC5, there is a separate isoform, ARPC5L, which can substitute for ARPC5 in the ARP2/3 complex. Previous studies have shown that these two subunits result in unique ARP2/3 complexes which drive different actin dynamics with regards to actin filament stability, depending on which isoform is in the complex (Abella et al. 2016). ARP2/3 complexes that contained ARPC5L resulted in actin filaments that dissembled ~2 fold more slowly than filaments made by complexes with ARPC5 (Abella et al. 2016). In contrast to the other ARP2/3 family members, ARPC5L expression in the TCGA melanoma dataset is more commonly under expressed than overexpressed (23/36 altered cases, **Figure 2.7A**). A comparison of the two isoforms reveal an approximately 2-fold increase in ARPC5 expression and a concomitant 2-fold

decrease in ARPC5L expression in tumor vs normal tissue samples (**Figure 2.7C**). Furthermore, examination of ARPC5 vs ARPC5L expression in benign nevus vs cutaneous melanoma in a separate melanoma dataset shows the same pattern (**Figure 2.7D**, (Talantov et al. 2005)). In summary, ARP2/3 complex members are often overexpressed in melanoma, but ARPC5L and ARPC5 have an inverse relationship with regards to expression levels.

2.5.6 Inhibition of YAP results in higher numbers of ARP2/3 complexes containing ARPC5L

We saw in our earlier results that ARPC5 protein levels decrease with YAP inhibition. In terms of ARPC5L, expression post YAP inhibition is not altered in the majority of lines examined, with the exception of a trend toward an increase in A375 cells (**Figure 2.5**). To investigate whether or not this decrease in ARPC5 expression could physiologically change the number of ARP2/3 complexes with ARPC5 as opposed to ARPC5L, we performed proximity ligation assays (PLA) with ARPC5L and ARPC2 (a complex member with no substitute) in mel537 and A375 cells treated with siYAP, siTAZ, siYAP/TAZ, or siARPC5. Quantification of the results revealed an increase in complexes with ARPC5L in YAP, YAP/TAZ, and ARPC5 inhibited cells ($p<0.05$, n = 100 cells/group). Collectively, this suggests that YAP predominantly drives expression of ARPC5 which results in higher number of ARP2/3 complexes containing ARPC5 in melanoma (**Figure 2.7E,F**).

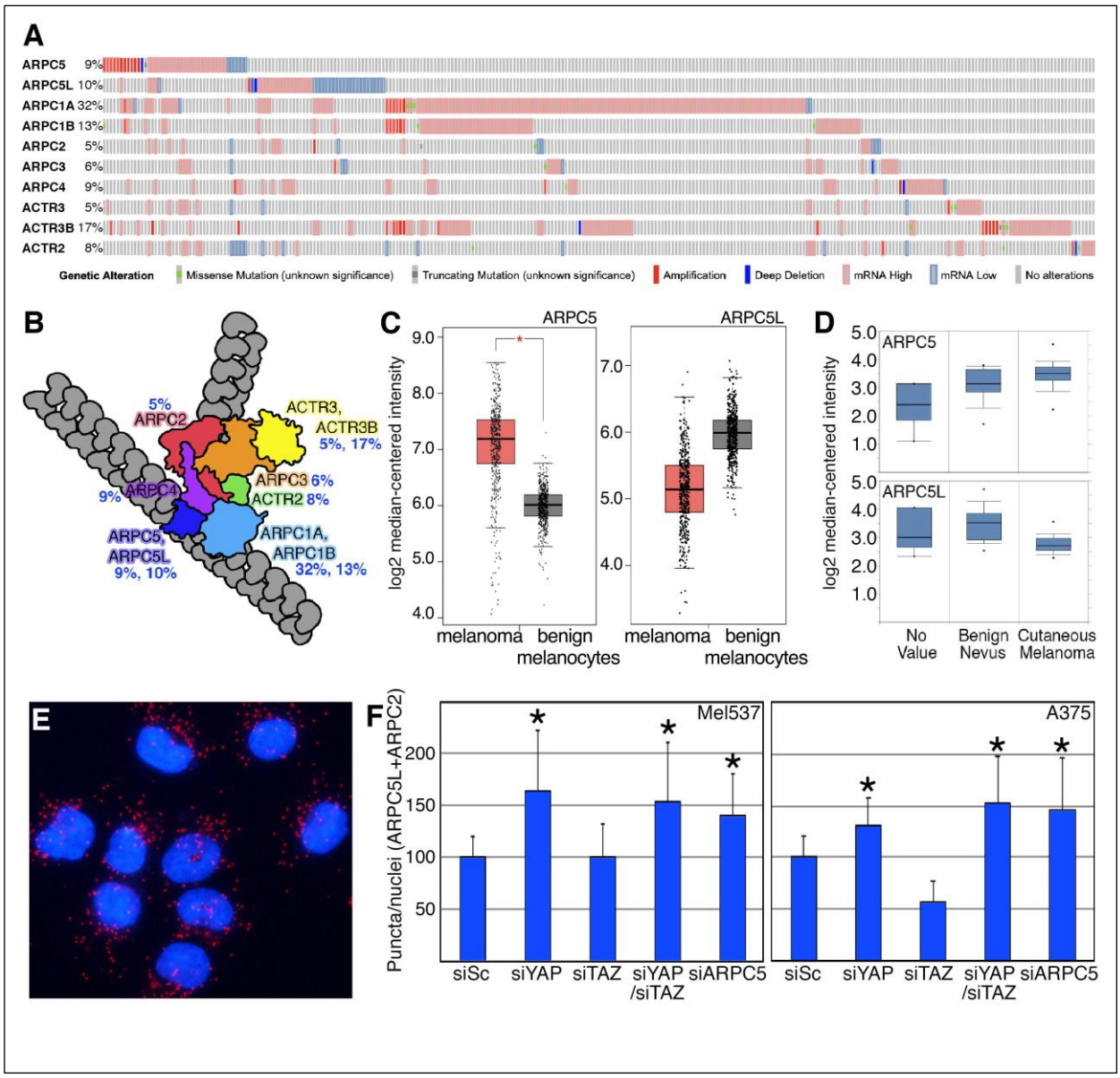

Figure 2.7: The relationship between ARPC5, ARPC5L in melanoma cells. (A,B) ARP2/3 subunits, except ARPC5L, are frequently overexpressed in human melanoma patients. Meta-analysis of the melanoma TCGA dataset for expression levels of ARP2/3 complex subunits from cBioportal (287 with ARP2/3 abnormalities of 471 total patients shown) represented as an oncoprint. (B). Representation of ARP2/3 complex on actin filament Isoforms are grouped by color and designated with percent of cases altered in human melanoma TCGA dataset. (C,D)

Figure 2.7 (continued) Expression of ARPC5 and ARPC5L is increased and decreased respectively in human melanoma. (C) ARPC5 GEPIA expression analysis of ARPC5 and ARPC5L transcripts comparing tumor to normal tissue from GTex and TCGA human melanoma datasets (D) Oncomine expression analysis comparing Benign nevus to Cutaneous melanoma samples for ARPC5 and ARPC5L (E-F) Proximity ligation assay (representative picture in E) on cells transfected with siRNA targeting YAP, TAZ, YAP/TAZ, and ARPC5. The results are quantified in (F), where average puncta per nuclei per group were counted (n=200). Values are mean $\pm$ SD. (*, $p < 0.05$).

2.6 DISCUSSION

YAP and TAZ are often dysregulated in many different types of cancers, where they drive expression of genes crucial for cancer initiation, progression, and metastasis (Zanconato et al. 2018; Zhao et al. 2008; Nallet-Staub et al. 2014; C. Y. Liu et al. 2017; Martinez et al. 2019; Guo, Kang, and Zhao 2018). In melanoma, inhibition of YAP and TAZ lead to decreases in invasion and lung colonies in nude mice tail vein injections (Nallet-Staub et al. 2014). In the context of drug resistance, PLX4032 resistant melanoma cell lines exhibited a gene signature similar to that of increased YAP activity, while YAP/TAZ inhibition led to lower viability in the presence of PLX4032 (M. H. Kim et al. 2016b). Furthermore increased YAP activity led to increased PLX4032 resistance (M. H. Kim et al. 2016b; Fisher et al. 2017). While these studies support the role of YAP in melanoma, the role that TAZ plays is not always directly addressed experimentally. It is possible that the two cofactors play unique roles specific for certain cancer processes. Here, we examine each factor and discover YAP-specific roles for melanoma migration and focal adhesion numbers.

Although genetic data indicates functional redundancy in some contexts, it should not be unexpected that these two proteins could have evolved unique functions, particularly given that YAP and TAZ contain unique moieties (Varelas 2014). Indeed, recent reports have started to delineate differential roles between these two transcriptional coactivators. YAP was found to be elevated in benign nevi and primary cutaneous melanomas as compared to normal melanocytes (X. Zhang et al. 2019). Furthermore, the same group found that in YAP inhibited conditions, TAZ is not able to compensate for YAP in the context of cell viability. In HEK293 cells, YAP was found to have greater impact than TAZ on cell proliferation and migration, among other cell functions (Plouffe et al. 2018). We find that YAP, but not TAZ, is the predominant player in our melanoma cell lines. Cancer Dependency Map Analysis (**Figure 2.1**), functional experiments (**Figure 2.2, 2.3**), and RNA-sequencing (**Figure 2.4**) all reveal a more substantial role of YAP than TAZ in their regulation of human melanoma progression. It is especially interesting that different cancers have unique and redundant roles for YAP and TAZ. A clue to the differential roles YAP and TAZ play may lay in the protein domains unique to YAP or TAZ. Due to the lack of DNA binding domain in YAP and TAZ, it is possible that YAP and TAZ have different binding partners specific to their unique binding domains to help facilitate their downstream effects. Future studies into protein domains specific to YAP, such as the SH3 and second WW domain, will be needed to fully understand how YAP drives migration and survival.

Here we find ARPC5 is downregulated with inhibition of YAP, but not TAZ. We show that direct inhibition of ARPC5 or YAP led to decreased cell migration and focal adhesion numbers. In head and neck squamous cell carcinoma, inhibition of ARPC5 decreased cancer cell migration and invasion (Kinoshita et al. 2012). In multiple myeloma, ARPC5 has been

implicated in its potential use as a biomarker for the disease (Xiong and Luo 2018). While those two studies and others have implicated a role of ARPC5 in driving cancer migration and invasion in other cancers, it has never been implicated in melanoma. Our data suggest that YAP is able to drive melanoma cell migration through transcriptional control of the ARP2/3 complex constituent ARPC5. Interestingly enough, the ARP2/3 complex has different properties with respect to the stability of the actin strands after nucleation when it contains ARPC5 vs ARPC5L (Abella et al. 2016). We find that ARPC5L does not change with either YAP or TAZ inhibition (**Figure 2.5**). Direct knockdown of ARPC5 phenocopies YAP inhibition in terms of decreased melanoma cell migration and focal adhesion numbers (**Figure 2.6**). As focal adhesion dynamics and cell migration are closely linked, it is possible that YAP controls focal adhesion turnover, dynamics, and stability through its regulation of ARPC5 (Nagano et al. 2012; Hoock et al. 2019). Furthermore, melanoma cells shift to larger numbers of ARPC5L-ARP2/3 complexes as compared to control than with inhibition of YAP or ARPC5, but not TAZ (**Figure 2.7**). We propose a model whereby YAP, but not TAZ, drives increased expression of ARPC5, in turn resulting in larger amounts of ARP2/3-ARPC5 complexes that result in a more migratory phenotype (**Figure 2.8**). The dynamic control of actin has been shown to control melanoma drug resistance, tumorigenesis, and even YAP/TAZ activity, among other functions (J. Kang et al. 2018; M. H. Kim et al. 2016b; Panciera et al. 2017). Further studies into how ARPC5 are needed to elucidate the mechanism of ARPC5-ARP2/3 driven metastasis.

In conclusion, this study provides evidence that YAP plays a more crucial role than TAZ in driving pro-tumorigenic phenotypes in melanoma cells, suggesting that YAP is a driver of melanoma progression, migration, and invasion. We provide support that YAP drives melanoma

migration through YAP specific regulation of ARPC5, shifting ARP2/3 dynamics towards a pro-migratory phenotype. Lastly, we postulate that melanoma is a great model to study differences between YAP and TAZ, as both biased and unbiased screens show a higher reliance on YAP in melanoma. While future studies will be needed to understand fully how YAP and TAZ differ, it is clear that these two genes have both overlapping and unique effects in driving their downstream effects.

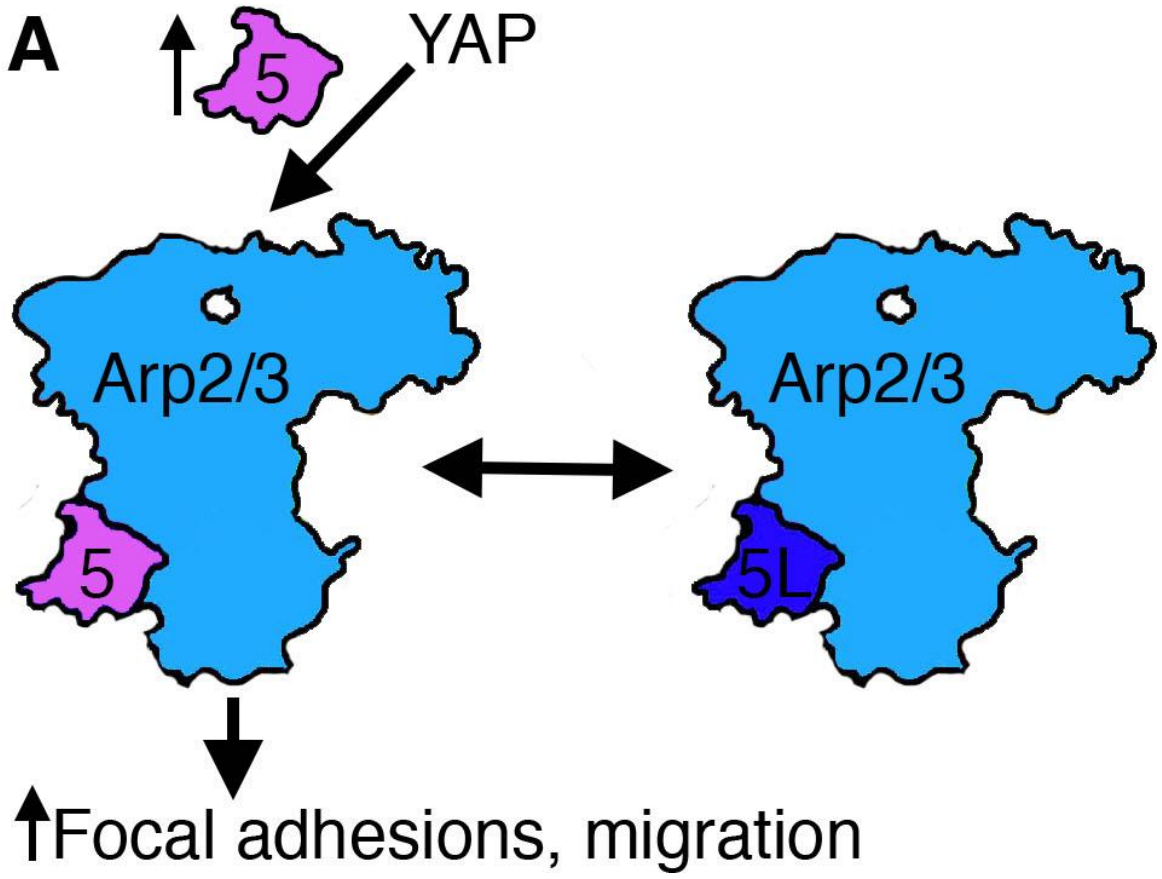

Figure 2.8: Proposed Model for YAP driven regulation of ARPC5 in melanoma migration. YAP drives expression of ARPC5. Increased expression of ARPC5 results in larger amounts of ARP2/3 complexes containing ARPC5 as opposed to ARPC5L. This shift in subunit results in increased numbers of focal adhesions and melanoma cell migration

CHAPTER 3: THE EFFICIENCY OF VERTEPORFIN AS A THERAPEUTIC OPTION IN PRE-CLINICAL MODELS OF MELANOMA

3.1 ABSTRACT

Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ-Binding Motif (TAZ) have gained notoriety for their ability to drive tumor initiation and progression in a wide variety of cancers, including melanoma. YAP and TAZ act as drivers of melanoma through its interaction with the TEAD family of transcription factors. Verteporfin is a benzoporphyrin derivative that is used clinically for photodynamic treatment of macular degeneration. Recently it has emerged as a potential inhibitor of YAP/TAZ-TEAD interaction independent of light activation. In this study we determine if verteporfin has clinical potential by testing this compound on human melanoma cell cultures and in a clinically significant mouse model, $Braf^{CA}$; Tyr-CreERT2; $Pten^{f/f}$, which parallels human melanoma in terms of disease progression, genetics, and histopathology. In culture, Verteporfin treatment induces a rapid drop in YAP and TAZ protein levels and cell numbers. In the transgenic model, utilizing drug levels that correspond to previously determined safe doses in human patients and with a dosing regimen calculated in this study, Verteporfin did not inhibit melanoma initiation or progression in comparison to mock treated controls. Taken together, our study suggests that although Verteporfin induces YAP/TAZ degradation in melanoma cell lines, Verteporfin was not effective as a YAP/TAZ-TEAD specific inhibitor of melanoma in our studies that aimed to mimic conditions found in clinic in terms of treatment regimen and disease model.

3.2 AUTHOR CONTRIBUTIONS

The majority of the work presented in this chapter was performed by myself. However, several individuals worked alongside me to conduct several of the experiments. Sixia Xiao helped to perform the immunohistochemistry found in **Figure 3.8**. Kelsey Ogomori helped to perform the Western Blot analysis found in **Figure 3.1**. Jon Hammarstedt helped to perform the scratch assay measurements found in **Figure 3.3**. Elizabeth Little taught me how to administer the small molecule inhibitor via intraperitoneal injection. This chapter was published in the Journal of Cancer (Lui et al., 2019).

3.3 INTRODUCTION

Melanoma is a tumor with an aggressive nature, high degree of metastasis and a rising incidence rate (Reed et al. 2012). Although significant discoveries have expanded therapeutic options in recent years, there are still many clinical challenges toward treating this disease. Yes Associated Protein 1 (YAP) and Transcriptional coactivator with PDZ-Binding Motif (TAZ) are two transcriptional coactivators that have been implicated to drive many different cellular processes that favor cellular proliferation, drug resistance, and metastasis in a wide variety of cancers, including melanoma (Nallet-Staub et al. 2014; Zanconato, Cordenonsi, and Piccolo 2016). YAP and TAZ were originally discovered as crucial members of the organ size controlling Hippo Pathway, where a central core kinase cascade regulates the location (and subsequently the transcriptional activity) of YAP and TAZ. In addition to the Hippo Pathway, recent studies have also shown YAP and TAZ to be regulated by a wide variety of Hippo independent signals (3). Both YAP and TAZ do not contain a DNA binding domain, so

formation of a complex with transcription factors to drive expression of downstream target genes is required. Traditionally their main partners in driving cancer progression and survival are the TEAD family of transcription factors (Zhao et al. 2008). Thus, it is possible that inhibition of YAP/TAZ-TEAD interaction could prove to be a viable therapeutic strategy against melanoma.

Verteporfin (market name Visudyne) is a benzoporphyrin derivative that has been traditionally used in the clinic for photodynamic treatment of macular degeneration (Liu-Chittenden et al. 2012). A recent *in vitro* screen yielded Verteporfin as a candidate inhibitor of YAP-TEAD interaction independent of light activation (Liu-Chittenden et al. 2012). Since then, many studies have shown that Verteporfin inhibits tumor volume, growth, and YAP expression in a wide variety of xenograft models (Gibault et al. 2016). While xenograft models in melanoma are a valuable tool for studying human melanoma cells and the process of metastasis in an *in vivo* environment, this mouse model is poorly predictive of clinical efficiency (Merlino et al. 2013). Transgenic models, with intact microenvironments and immune systems, are a better predictor of translational outcomes for human patients (Day, Merlino, and Van Dyke 2015; Olive et al. 2009). Prior to these studies, Verteporfin use has not been examined in cutaneous melanoma. Therefore, the ability of Verteporfin to inhibit melanoma growth and survival was tested. The response to Verteporfin by a panel of human melanoma cell lines in culture in terms of YAP and TAZ protein levels, cell growth, migration, and cellular morphology was measured. In addition, Verteporfin was tested as a therapeutic agent for melanoma in a pre-clinical transgenic model, *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ mice, following a determined dosing regimen at clinically relevant drug levels.

3.4 MATERIALS AND METHODS

Cell culture and growth curves

Human melanoma lines A375, LOX IMVI, A375-P, A375-M, mel-537, mel-624, SKMEL5, SKMEL23 and SKMEL28 (ATCC, Manassas, VA and University of Chicago Comprehensive Cancer Center Core Facilities) were cultured in DMEM with 10% FBS (Sigma-Aldrich). A375M and A375P refer to selected cell lines derived from A375 cells that exhibit low and high levels of metastasis for the A375 cell line *in vivo* (Kozlowski et al. 1984). Morphology, melanoma-marker testing, and histological analysis were used to verify melanoma cell identity and lack of mycoplasma contamination. For cellular growth curves, Cells were initially seeded at 10-20% and images in 5 random locations were taken daily. Cell numbers were calculated by averaging the daily cell counts for each of the 5 images per group over the course of 0 to 8 days in various conditions (Verteporfin treatments, DMSO treatments, YAP/TAZ knockdowns and transfection with siScramble controls). All growth curve experiments were performed minimally in triplicate. Normalization of curves was performed by calculating fold change levels over starting cell numbers from day 0.

Western analysis

Cells were lysed in RIPA buffer and 30 μg total protein was separated on 4-15% Bis-Tris gels and subsequently transferred to nitrocellulose membranes. The membranes were then probed overnight with 1:1000 YAP/TAZ antibody (Cell Signaling) and 1:10000 GAPDH (Cell Signaling). Membranes were washed with 1X TBS-T three times for 20 minutes and incubated

with 1:4000 goat anti-rabbit IgG-HRP (Santa Cruz) and developed with Clarity Western ECL substrate according to the manufacturer's instructions (Bio-Rad).

Verteporfin timecourse

Cells were treated with Verteporfin (Sigma-Aldrich) at concentrations of 1, 2, 5 μM, or DMSO carrier alone. Cells lysates were collected at various time points (30 minutes, 2 hours, 3 hours, 24 hours) and analyzed for YAP and TAZ levels via Western blotting. Verteporfin treated samples were compared to DMSO vehicle control treated cells.

SiRNA treatment

Cells were seeded at 50-70% confluency in 6-well plates and subsequently transfected with 5μl of a 20μM siRNA stock solution against YAP1, WWRT1 (TAZ), and/or siScramble (Thermofisher ID Number S20366 - YAP1, S24787 - WWTR1, 4390844 - siScramble) using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions. Cell lysates were collected 2 days post-siRNA transfection.

Scratch assays

Scratch assays were performed and analyzed following prior methods (Liang, Park, and Guan 2007; H. Lee, Kim, and Kim 2012). Cells were seeded at full confluency in 6 well plates. After siRNA transfection a wound was created using a 10 μM sterile tip. The media was then replaced with DMEM (10% FBS) with or without 2 μM Verteporfin. Photographs were taken immediately after wound creation and 24 hours post scratch. The open area was measured in

arbitrary units using the ruler tool in Adobe Photoshop CS6. Percent closure for each picture pair (0 hr and 24 hr) was calculated as (gap at 24 hr/gap at 0 hr X 100). For each group (experimental and control) and cell line, the experiments were performed minimally in triplicate. For graphs shown in Figure 3, the level of closure for each control cell line at 24 hr is set to 100% to normalize the data across cell lines.

Cell length and morphology

Cells were treated with Verteporfin (2 μg/ml), siYAP/TAZ, or DMSO alone (2 μg/ml). Cell length was defined as the length from the tip of the longest dendrite to the cell body to the tip of the second longest dendrite. Length was then measured in arbitrary units for 50 cells in each group using the ruler tool in Photoshop CS6.

Mouse Verteporfin treatments

Braf^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ mice were previously described (Dankort et al. 2009). For localized melanoma induction, topical administration of 1-2 μl of 1.9 mg/ml (5mM) 4-hydroxytamoxifen (4HT) was applied on three consecutive days to 12 week old *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ mice. The mice were subjected to 4 and 6 mg/kg Verteporfin intraperitoneal injections every other day for the course of the study starting the first day of melanoma induction. An equivalent amount of DMSO was used as a vehicle control. Tumors were collected 42 days post induction.

Mouse Verteporfin kinetics

The following protocol was modified from previous methods (Busetti et al. 1999). Wildtype mice (6 mice/group) were subjected to 0, 2, 4, 6 mg/kg Verteporfin intraperitoneal injections. Mouse tissue samples were collected 6, 24, 48, and 72 hours post IP-injections. The samples were then homogenized in 2% SDS, diluted tenfold with a chloroform-methanol binary mixture (1:2 v/v), and centrifuged for 10 min at 3000 RPM. The resulting supernatant was then measured using fluorescent spectroscopy with excitation settings at 400 nm and emission at 550-750 nm.

Immunohistochemistry

Skin tumor samples were harvested from mice 42 days post induction. Tumor samples were fixed with formalin and subsequently paraffin embedded. Tissue was cut into 5μM slices and rehydrated through an ethanol to water wash series. Antigen retrieval was performed by boiling the sections in Tris-EDTA buffer for 30 minutes and subsequently placed in cold ddH_2O for 10 minutes. Sections were blocked using 5% normal horse serum in 1X TBS and probed for YAP/TAZ (Cell Signaling, 1:200) at 4 degrees Celsius overnight. Sections were washed with 1X TBS-T and incubated with DyLight 594 Anti-Rabbit IgG (diluted in blocking buffer 1:200, Vector Labs) at room temperature for an hour. Three additional washes with washing buffer for 5 minutes were performed prior to mounting with Vectashield Anti-fade Mounting Medium with DAPI (Vectashield).

Densitometric analyses of YAP/TAZ expression

To quantify levels of expression of YAP and TAZ in experimental and control group tumor specimens, 10 images were taken from 3 independent slides where YAP and TAZ expression were detected by immunofluorescence analysis. For each image, densitometry of the resultant fluorescence was performed with ImageJ software (ImageJ version 1.47 public domain software; National Institutes of Health, Bethesda, MD, USA). For normalization of the values, each densitometric reading for YAP/TAZ (red channel) was divided by the value for the nuclear DAPI staining from the blue channel. The data presented are densitometric readings from the averages from the three independent slides per group.

Statistical analyses

Significance of the differences between the control and experimental groups was determined with Student's t-test and Chi-square analysis with a confidence interval of 95%. All values stated as significant have p values of less than or equal to 0.05 unless indicated. All experiments were performed minimally in triplicate.

3.5 RESULTS

3.5.1 YAP and TAZ are expressed in melanoma cells, and this expression is reduced by Verteporfin

To initially determine the effect of Verteporfin on YAP and TAZ, 9 human melanoma cell lines were analyzed for the presence of YAP and TAZ proteins. 7 lines expressed varying

degrees of both YAP and TAZ protein, while 2 lines expressed either YAP or TAZ (Figure 1A). Three lines (A375, SKMEL5, mel-537) were chosen for further analysis based on their varying degrees of YAP and TAZ expression. The benzoporphyrine derivative, Verteporfin (Figure 1B), was identified as an inhibitor of YAP and TAZ function (Liu-Chittenden et al. 2012). Verteporfin treatment (2 μM) leads to a decrease in both YAP and TAZ protein levels that was detectable as early as 30 minutes and up to 24 hours (**Figures 3.1C,3.1D**).

3.5.2 Both Verteporfin treatment and direct RNA-interference of YAP and TAZ inhibits cell population expansion in melanoma cells

YAP and TAZ, as downstream effectors of the HIPPO signaling pathway, are implicated in controlling cellular proliferation and organ size in humans and flies (J. Huang et al. 2005; Dong et al. 2007). To determine if Verteporfin affects melanoma cell growth, cell numbers were were recorded over a time course with exposure to the drug or with carrier alone (DMSO). Verteporfin treatment at 2 different concentrations (2 and 5 μM) led to a reduced rate of melanoma cell proliferation over a time-course of several days as compared to vehicle control (**Figure 3.2A**). While each cell line exhibited multiple population doublings in control groups, the rate of cell proliferation in Verteporfin-treated groups did not significantly rise above starting cell numbers in the three lines tested. Here, we find that Verteporfin significantly inhibits cell growth in A375, mel-624, and mel-537 melanoma cells at concentrations of 2 and 5μM. Since Verteporfin treatment led to a reduction of cellular growth and YAP/TAZ protein levels, and that Verteporfin has been previously described to inhibit YAP and TAZ function (Liu-Chittenden et

al. 2012), inhibition of YAP and TAZ through siRNA targeting was performed to determine if the resulting cellular phenotype would replicate that of Verteporfin treatment. Multiple

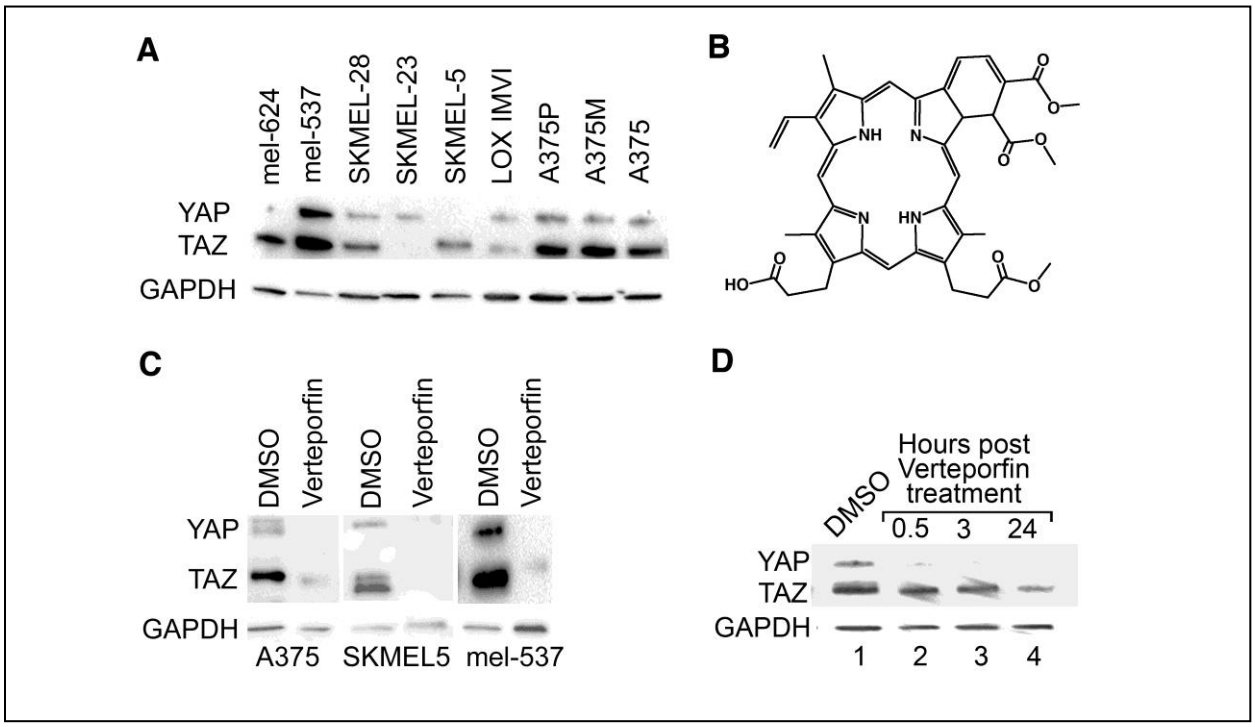

Figure 3.1: Verteporfin treatment decreases YAP and TAZ protein levels in melanoma cells. (A) YAP and TAZ are expressed in melanoma cells. Western blot analysis probing for YAP and TAZ, with GAPDH as a loading control, was performed in a panel of cell lines (Lanes 1-9). (B) Chemical structure diagram of Verteporfin, as modified from information from manufacturer (Sigma-Alrich). (C) YAP and TAZ protein levels drop upon Verteporfin treatment. A375, SKMEL5, and mel-537 cells were treated with Verteporfin at 2 μM. DMSO was used as a vehicle control. 2 hours after treatment, cell lysates were collected for Western blot analysis. (D) A375 cells were subjected to Verteporfin treatment (2μM) and cell lysates were collected 30 minutes, 3 hours, and 24 hours post treatment for Western analysis testing for YAP and TAZ expression, with GAPDH as a loading control.

YAP and TAZ specific siRNAs were tested for specificity and efficiency in inhibition (**Figure 3.3**), and siTAZ2 and siYAP4 were utilized for all following experiments. These siRNAs inhibit

>90% of each protein, as well as both factors when combined (**Figure 3.2B**). In parallel with the findings for Verteporfin, inhibition of both YAP and TAZ lead to a significant reduction in the rate of melanoma cell proliferation as compared to the siScramble control group (**Figure 3.2C**). Block of both YAP and TAZ expression resulted in cell numbers of <50% and <10% in A375 and mel-537 cells, respectively, in comparison to siScramble control groups, as determined by quantification of cell numbers in at least 5 cell fields/group (100X magnification). Both Verteporfin treatment and inhibition of YAP and TAZ by siRNA resulted in a significant attenuation in cell population expansion in A375 and mel-537 cells.

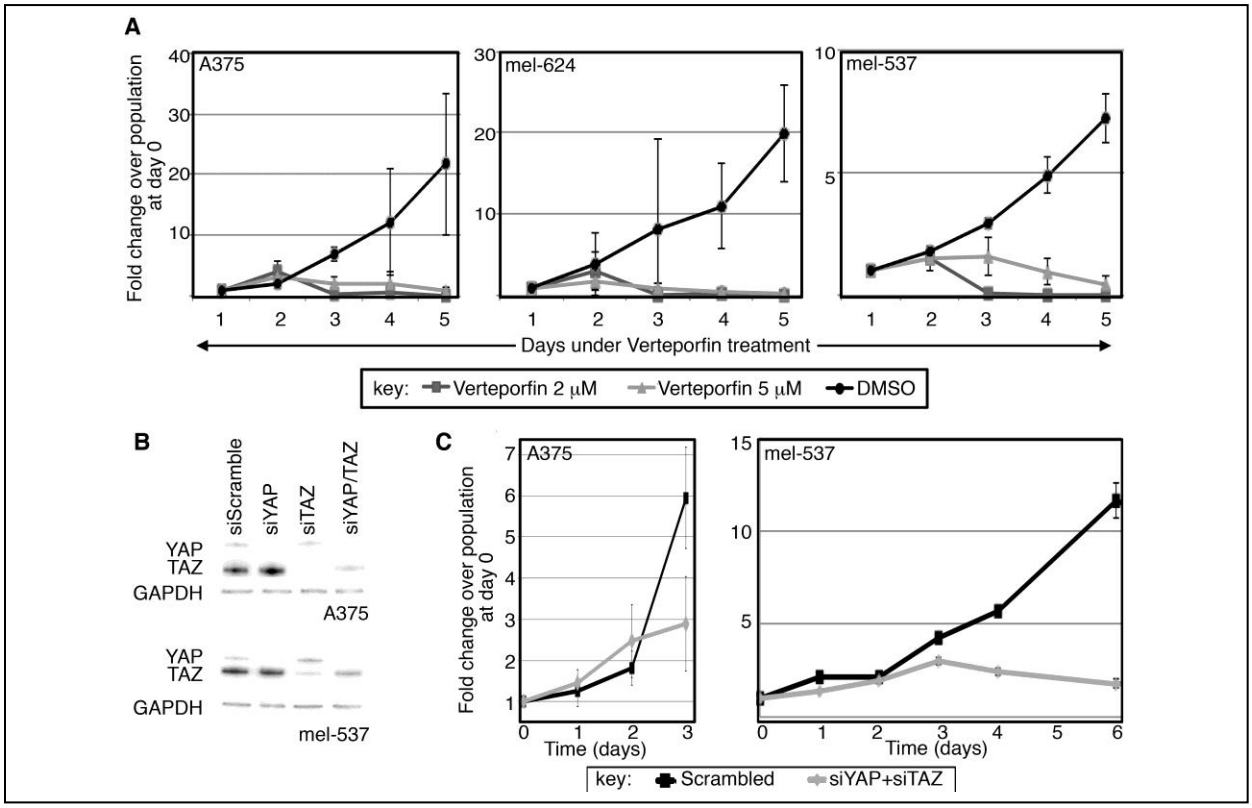

Figure 3.2: Verteporfin treatment and direct RNA-interference of YAP and TAZ inhibits cell population expansion. (A) Verteporfin treatment reduces the rate of melanoma cell proliferation.

Figure 3.2 (continued) SKMEL5, mel-624, and mel-537 cells were subjected to Verteporfin treatment (2 and 5 μM) and cell counts were taken over the course of 5 days. There are significant differences between control and experimental groups at 5 days of treatment ($p<0.05$). Cells from at least five independent fields (100X magnification, with approximately 10-500 cells/field depending on experimental group and conditions) for each experiment were counted, and experiments were performed in triplicate. Graphs are plotted as fold change in overall cell numbers (y axis) over time (x axis). (B) Gene-specific targeting of YAP, TAZ, or both in melanoma cells. Western analysis probing for YAP and TAZ, with GAPDH as a loading control, was performed in A375 (top panel) and mel-537 (lower panel) cell lines. Expression of both YAP and TAZ is inhibited by >90% of control levels as measured by densitometry for both cell lines (A375 and mel-537). (C) Gene-specific siRNA against YAP and TAZ lead to reduced melanoma cell numbers when compared to cells transfected with siScramble negative control siRNAs. There is a significant difference in cell numbers at day 3 (A375) or Day 6 (mel-537)($p<0.05$). Cell counts were performed following methods described in (A).

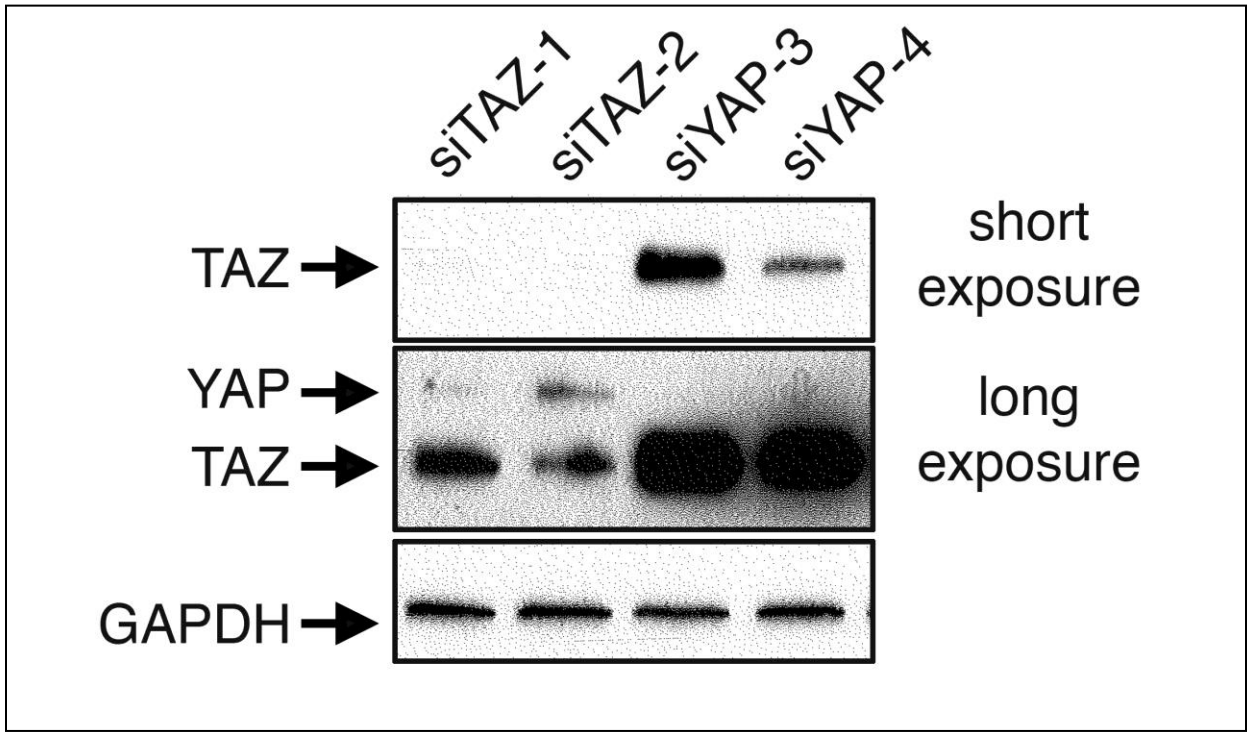

Figure 3.3: Multiple YAP and TAZ targeted siRNAs are specific for YAP and TAZ. Western analysis for YAP and TAZ protein levels in A375 cells transfected with TAZ-specific (siTAZ-1, siTAZ-2) or YAP-specific (siYAP-3, siYAP-4) siRNA. Antibody for YAP/TAZ

Figure 3.3 (continued) recognizes both proteins, but a longer exposure is required to sufficiently visualize YAP (long exposure) in comparison to TAZ (short exposure). Western membranes were stripped and reprobed with an antibody recognizing GAPDH to function as a loading control.

3.5.3 Cellular response between Verteporfin treatment and direct RNA-interference of YAP and TAZ is divergent in terms of cellular morphology

Other functional tests, including migration assays and cell length quantification, were performed on cells treated with Verteporfin and YAP/TAZ siRNA treated cells. To test migration, wound healing assays were performed, and the scratch area was measured right after wound creation (time 0). At set times after the procedure (depending on cell line) the distance of cells migrating into the area was measured. Two sets of experiments were run, one with Verteporfin and DMSO as a control (Figure 3A), and siYAP/TAZ and siScramble as a control (**Figure 3.4B**). To normalize findings between cell lines, gap closure of control cells at 24 hours post scratch formation is set at 100% control closure levels. Both experiments showed similar but not identical trends. Verteporfin inhibited migration in 4/6 cell lines. One of the lines with a significant attenuation in migration by Verteporfin, A375, had a similar trend with siYAP/TAZ, albeit not to significant levels. In addition, mel-537 cells did not demonstrate any significant migratory change with Verteporfin treatment, but migration of this cell line was significantly inhibited with siRNA interference of YAP and TAZ.

Cell morphology and length was also analyzed between Verteporfin treated cells and cells with YAP and TAZ inhibition with siRNA. Overall, the cells had morphological differences between all groups. In comparison to controls, Verteporfin treated cells were rounded and

appeared to have vesicles, while the siYAP/TAZ treated cells were longer and linear/bipolar rather than epithelioid/polygonal (**Figure 3.5A**). The siYAP/TAZ treated cells were significantly longer than control cells, while Verteporfin treated cells were not. The overall cell length was

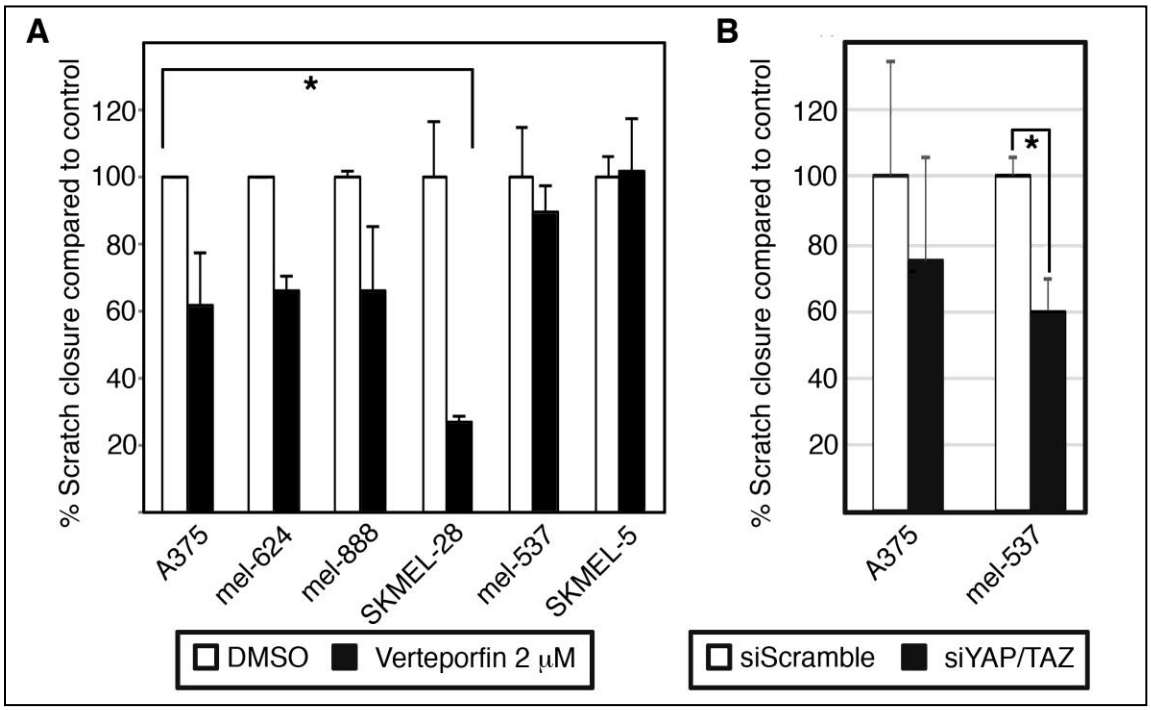

Figure 3.4. Both Verteporfin treatment and siRNA inhibition of YAP and TAZ inhibit melanoma cell migration. (A) Wound healing assay in Verteporfin (2μM) treated melanoma cells. The percent of wound closure was measured by dividing the width of the initial wound with the width of the wound 24 hours post scratch for the various groups comparing Verteporfin treated cells to control in 6 different melanoma lines. DMSO was used as a vehicle control. (B) Wound healing assay in YAP/TAZ siRNA inhibited melanoma cells. The percent of wound closure 24 hours post wound was measured comparing YAP/TAZ knockdown cells to siScrambled control in A375 and mel-537 cells. For both panels, an asterisk (*) indicates $p<0.05$, or $p<0.005$ for SKMEL-28 cells.

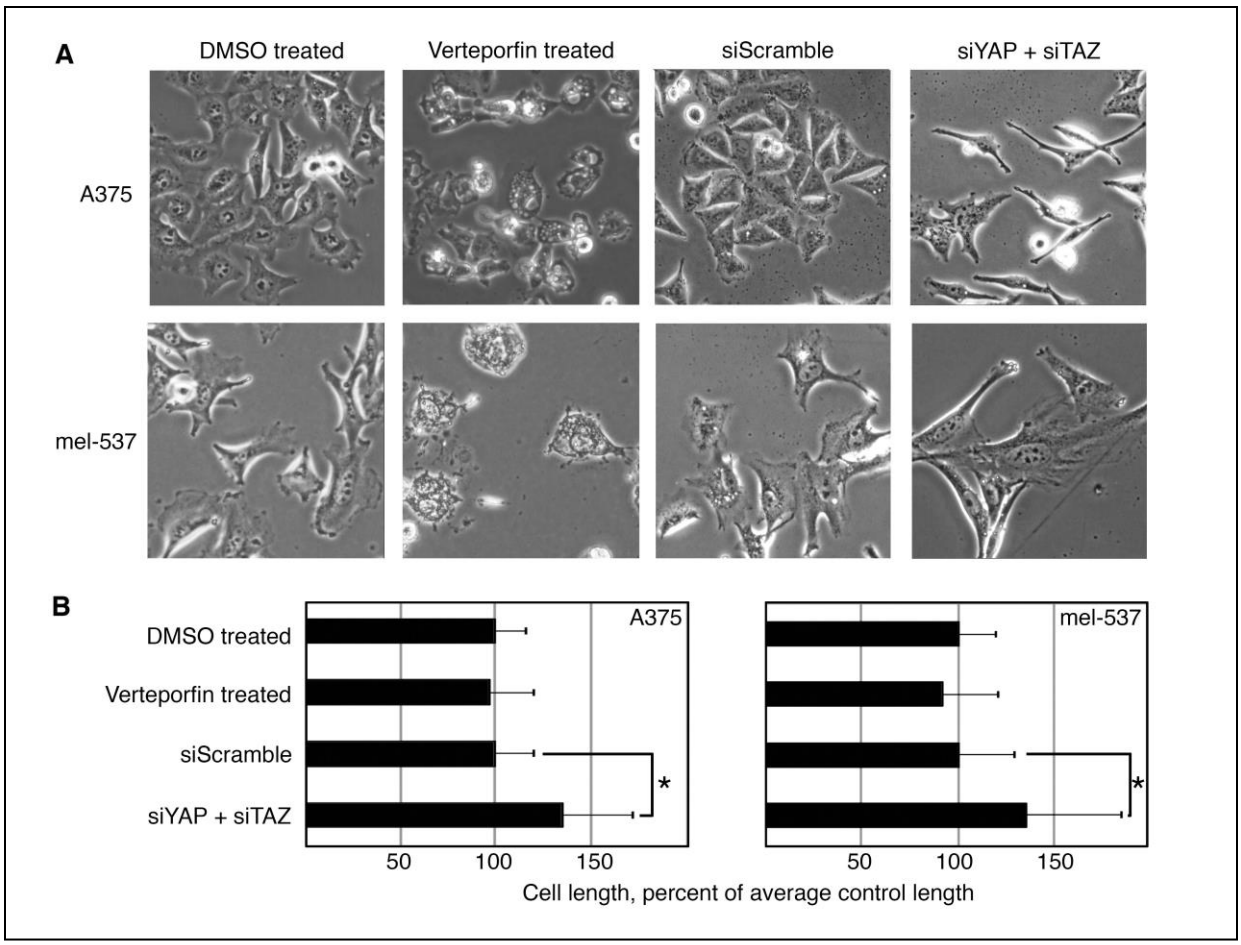

Figure 3.5: Verteporfin treatment does not phenocopy dual YAP/TAZ knockdown. (A, B) YAP and TAZ loss through siRNA inhibition, but not Verteporfin treatment, induced elongated dendritic extensions. Dendritic extension length was measured in Verteporfin treated (2μM) or YAP/TAZ siRNA inhibited in A375 and mel-537 cells 2 days post treatment. DMSO vehicle and siScramble treatments were used as controls. Examples of overall cellular morphology (A) and quantification of dendritic extensions (B) where average control cell length is set at 100% cell length. For each group, at least 200 cells/group was measured. Only the siYAP + siTAZ cell group demonstrated a significant length difference when compared to its matched control group siScramble (both cell lines $p<0.05$ as indicated by an asterisk (*)).

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measured as the distance from the distal points from the longest dendritic-like process to the cell body and then again to the next longest process (**Figure 3.5B**). After siRNA induced YAP/TAZ

knockdowns, both A375 and mel-537 cells increased processes to 134.2% ± 36.8% and 135.1 ± 48.8% respectively as compared to all other groups (DMSO, Verteporfin, siScramble, n = 200 cells/group, $p<0.05$). While there are similar trends between Verteporfin treated cells and cells with YAP/TAZ inhibition due to siRNA interference, there are also some differences in the cellular response including migration, morphology, and cell length.

3.5.4 Verteporfin treatment of a mouse model of melanoma does not result in a significant change in tumor initiation, progression, or overall tumor size

Verteporfin treatment was tested in an *in situ* transgenic model of melanoma. For this pre-clinical study, parameters were set utilizing a model that has similar tumor progression and genetics to the human disease, and with drug dosing that follows levels outlined for prior human clinical trials and usage in patients. For the mouse model, the *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ transgenic mice, where topically administered tamoxifen induces expression of a mutant Braf allele and deletes Pten expression (Dankort et al. 2009), was selected to induce *in situ* melanoma formation (**Figure 3.6**). This model mimics human disease genetically, histologically, and in the kinetics of disease progression. After three treatments of tamoxifen topically, the model typically develops pigmented lesions in approximately two weeks that quickly progresses to large nodular tumors within the following 2-4 weeks (Hooijkaas et al. 2012; Dankort et al. 2009). In terms of dose, amounts were derived from prior studies for human use, and equivalent levels were given to the mice that were determined to be safe in humans in the clinic, following guidelines outlined by the Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP) study

group (Bressler 1999) and in clinical trials for eye disease and cancer (Bressler and Bressler 2000; Huggett et al. 2014). In these studies, it was determined that optimal dose was 6 mg per square meter of body surface area (but up to 12 mg/m^2 was tolerated). Following prior methods for conversion between mg/m^2 to mg per kg of body weight (Schein et al. 1970; Freireich et al. 1966), this converts to 0.2-0.4 mg/kg for humans, or 2-4 mg/kg for mice.

To properly test Verteporfin as a therapeutic *in vivo*, we tested the kinetics of Verteporfin in the C57B6 mouse strain. Following previously published methods (Busetti et al. 1999) we first identified optimum absorbance for detecting Verteporfin in tissues (680 nm) as well a corresponding spectral peak for background and low Verteporfin absorbance (540 nm). Readings were normalized by dividing Verteporfin readings at 680 nm by background levels at 540 nm (**Figure 3.7**). To test penetrance and durability of Verteporfin in mouse tissues, mice were treated with three doses of drug (2, 4, and 6mg/Kg) and liver and skin tissues were collected over a time course of 6, 24, 48 and 72 hours. Verteporfin levels in both the skin and liver dropped quickly in the first 24 hours but were still present for up to 3 days post injection (**Figure 3.7**). Based on these control experiments, a drug treatment regimen of intraperitoneal injections every other day at 2 different Verteporfin concentrations was performed. Under the conditions tested, Verteporfin treatment did not inhibit tumor initiation as compared to the DMSO control (**Figure 3.8A**). Verteporfin and DMSO treated control mice were observed daily for the presence of pigmented lesions at the location of tamoxifen treatment on shaved back skin. Both experimental and control groups developed nevoid-like growths between 18 to 21 days post tamoxifen induction, with insignificant differences in nevi appearance between the 4 and 6

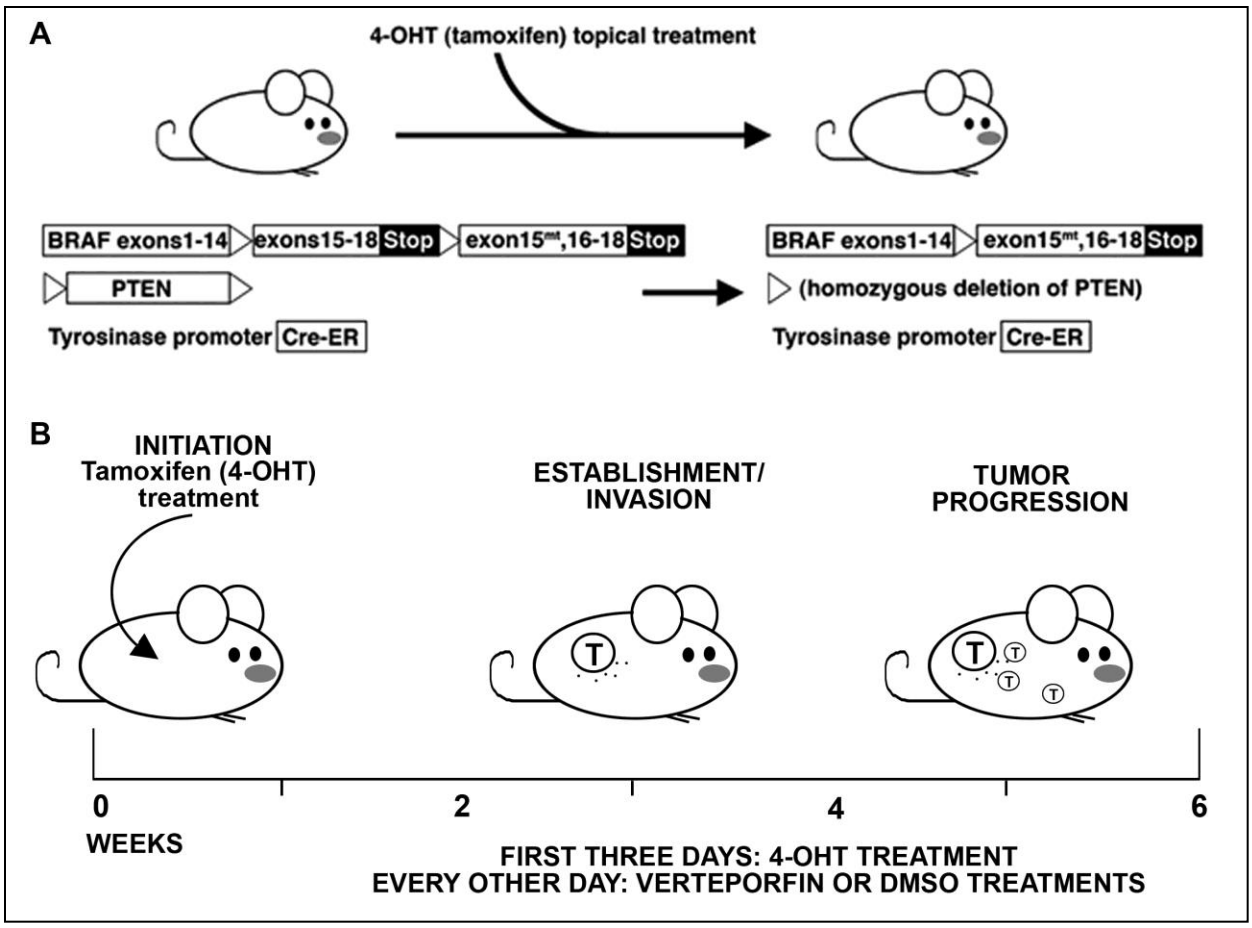

Figure 3.6. Summary schematic of *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ murine melanoma model experiments. (A) Treatment and transgenes in the *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ model. 4-hydroxytamoxifen (4HT) is applied topically, which deletes PTEN while simultaneously inducing expression of a constitutively active BRAF, inducing melanoma in the mouse. (B) Schematic of timeline for experimental procedure and kinetics of tumor initiation and progression.

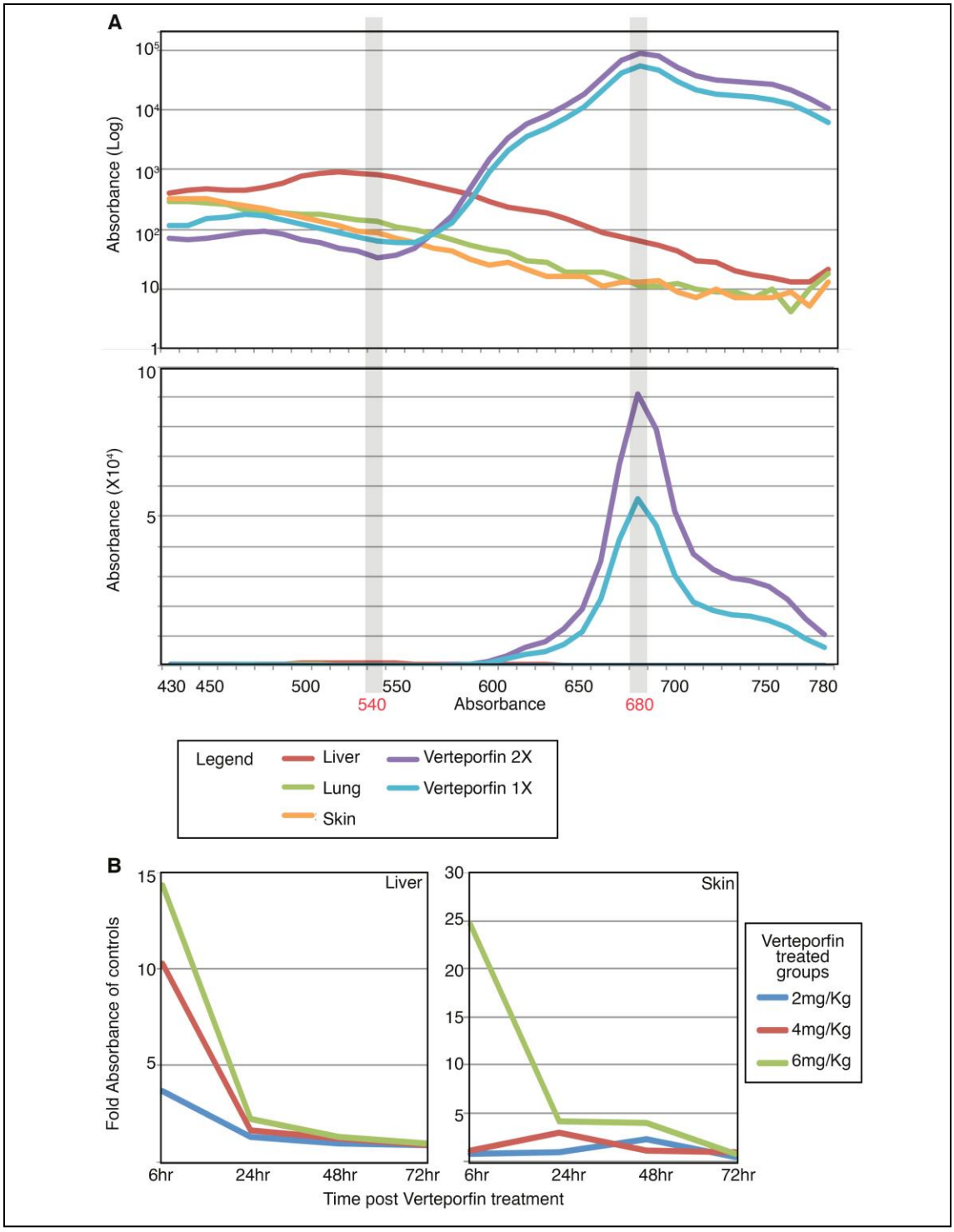

Figure 3.7: Verteporfin is detectable in the skin for up to 48 hours. (A) Spectral curves of wild-type untreated tissues (liver (red line), lung (green), skin (orange) and two different concentrations of Verteporfin (1 (1X, blue line) and 2 (2X, purple) mg/mL) indicate high

Figure 3.7 (continued) Verteporfin absorbance/low tissue background at 680 nm and low Verteporfin absorbance/high tissue background at 540 nm. Readings are displayed as absorbance over wavelength, shown with absorbance plotted as log (upper graph) and linear (lower graph) scale. (B) Verteporfin levels are still present 3 days post injection. Wildtype mice (6 mice/group) were subjected to 0 (control group), 2 (blue line), 4 (red), 6 (green) mg/kg Verteporfin intraperitoneal (IP) injections. Mouse tissue samples were collected 6, 24, 48, and 72 hours post IP injection. The levels of Verteporfin (readings at 680 nm) were normalized against tissue background (at 540 nm) and plotted against time post-injection prior to tissue collection.

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mg/Kg groups and the control mice using either the Mantel-Cox log-rank test (P = 0.3462) or the Gehan-Breslow-Wilcoxon test (P = 0.5597). In our system, there was no significant difference in the progression of tumors in terms of overall tumor size. After tumor initiation, the pigmented lesions develop into nodular melanoma in this mouse model in approximately 4 weeks after tamoxifen induction (**Figure 3.8**). At 45 days, the tumors grew to nodules of approximately $1cm^3$ and mice were sacrificed and tissue was harvested. The gross appearance of tumors was similar between groups (example shown in **Figure 3.8B**). Mouse tumors were measured with a caliper externally and volumes were calculated using the formula $V= a X b^2/2$, where a is the largest diameter and b in the smallest (24). Tumor sizes are normalized to percent of the average size for control (DMSO treated) tumors. There was not a significant difference in tumor size between treatment and control groups (p=0.262, **Figure 3.8C**).

Levels of YAP/TAZ were detected in the collected tumors. Immunohistochemical staining for YAP/TAZ shows no reduction of YAP/TAZ levels in Verteporfin samples as compared to DMSO control (**Figure 3.8D**). To quantify the immunofluorescent staining, YAP/TAZ (red channel) was measured by densitometry using image analysis (ImageJ) from 3 samples and 10 separate images per sample, and the values were normalized to DAPI nuclear

stain fluorescence (blue channel). The difference in the fluorescent staining between experimental and control groups was not significant (p=0.326, **Figure 3.8E**).

In summary, we provide evidence that although Verteporfin induces both a rapid drop in YAP/TAZ protein levels and a reduction in melanoma cell numbers in culture, it does not inhibit melanoma tumor initiation and progression *in vivo* in *Braf*^{CA}, *Tyr-CreERT2*, *Pten*$^{f/f}$ mice. These data suggest that a role for Verteporfin as a candidate for melanoma therapeutics is limited.

3.6 DISCUSSION

YAP and TAZ make attractive targets for melanoma therapy due to their roles in melanoma progression. Recent reports implicating Verteporfin as a molecule that targets and disrupts the function of these proteins make this small molecule inhibitor an attractive melanoma therapeutic candidate. While our data and others support an inhibitory role of Verteporfin on YAP/TAZ proteins in culture and in orthotopic models, this drug was not effective in the *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ transgenic model of melanoma. The work presented here demonstrates some in culture effects of Verteporfin on tumor cells that mirror YAP/TAZ inhibition by siRNA (**Figure 3.2**). However, there are also some notable differences. Most notably, there was a significant divergence in outcomes on cellular phenotype and cell length. While siRNA targeting YAP and TAZ induce an elongated bipolar cell morphology, this was not seen with Verteporfin treatment over a wide span of drug concentrations (0.1 ng to 10 μM) (**Figure 3.5**). An even more significant difference between our studies and prior published works is the reporting of significant *in vivo* anti-tumor properties of Verteporfin while our findings do not. While the findings in these other papers record significant findings, we believe that the lack of response

Verteporfin treatment in this report (**Figure 3.8**) is due to differences in our experimental approach. The rationale for this conclusion is 1) the mouse models used in the studies, 2) the methods used to calculate proper dosage of drug, and 3) drug delivery route. Our studies, as designed, should more faithfully predict how Verteporfin would function in clinic as a drug treatment of melanoma.

To conduct the experiments presented here, a transgenic model of melanoma was employed since the *in situ* initiation, tumor progression, disease kinetics, and histopathology more faithfully mimic human melanoma (Becher and Holland 2006; Dankort et al. 2009) than the immunodeficient transplant models used for other published studies (Feng et al. 2014; Jiang et al. 2015; Nguyen et al. 2015; Slemmons et al. 2015; Song et al. 2014; Yu et al. 2014). This difference between models may reveal some insight into the mechanism of action of Verteporfin. While we saw no effect against melanoma tumors in our model (**Figure 3.8**) there is no doubt that there is a measurable and significant consequence of Verteporfin treatment in the transplant models. It may be that some (or all) of the responsive cells are not the tumor cells at all but the supportive vasculature. Verteporfin is demonstrated to inhibit angiogenesis and lymphangiogenesis in both eye diseases and cancer models (Tatar et al. 2007; Hou et al. 2017; Wei et al. 2017). The disparity in findings may be due to different vascular requirements of tumors formed *in situ* in a native environment versus a large mass of transplanted tumor cells. Another clue is the impressive findings of Verteporfin use in uveal melanoma models (Feng et al. 2014; Yu et al. 2014). One of these studies, unlike the other published works that rely on xenograft immunodeficient mouse models, uses a clinically relevant orthotopic transplant to the vascular rich environment of the eye (Yu et al. 2014). It is not clear if the results are the

consequence of YAP/TAZ inhibition of the transplant cells or from Verteporfin influencing the surrounding vascular cells, and if YAP/TAZ is or is not the direct target within these vascular cells. Indeed, there are reports that find the action of Verteporfin to be YAP/TAZ independent and our studies support these findings (Huabing Zhang et al. 2015; Dasari et al. 2017).

Another major difference in the experiments presented here and other studies are the levels of Verteporfin used to treat the mice, as well as the delivery of the drug. As we focused on designing a murine treatment plan that would faithfully predict how Verteporfin could function clinically as a melanoma therapeutic, we decided to utilize the mouse equivalent dosing of Verteporfin that is used in clinic. This is in stark contrast to previously published works, which use 200-2000X the levels of drug over that what is tested as safe in humans. In support of this, many of the published studies report a decrease of weight of the experimental mice in comparison to controls. While this may be due, as proposed, to reduced tumor burden, it may also be a sign of cachexia. The dosing in the studies presented here follow levels deemed safe for humans clinically and calculated to the equivalent dosing in mice (Bressler 1999). In addition, while other studies inject Verteporfin or related compounds directly into or proximal to the tumor site, the studies presented in this report relied on systemic treatment. The rationale for this approach is that for clinical efficiency, it is necessary for compounds to travel through the body in a biologically active form and reach tumors. Melanoma may present with a primary cutaneous site, but often there are multiple metastases and these secondary tumors are linked to the morbidity and mortality of this cancer (Zbytek et al. 2008). We find that the drug reaches distal sites (**Figure 3.7**), but may be an inactive metabolite, since YAP/TAZ levels are unaffected in the tumors (**Figure 3.8**). While our studies do not support Verteporfin as an option for

melanoma therapy, our studies and others do support that Verteporfin has potential beyond its current use as a photodynamic therapeutic.

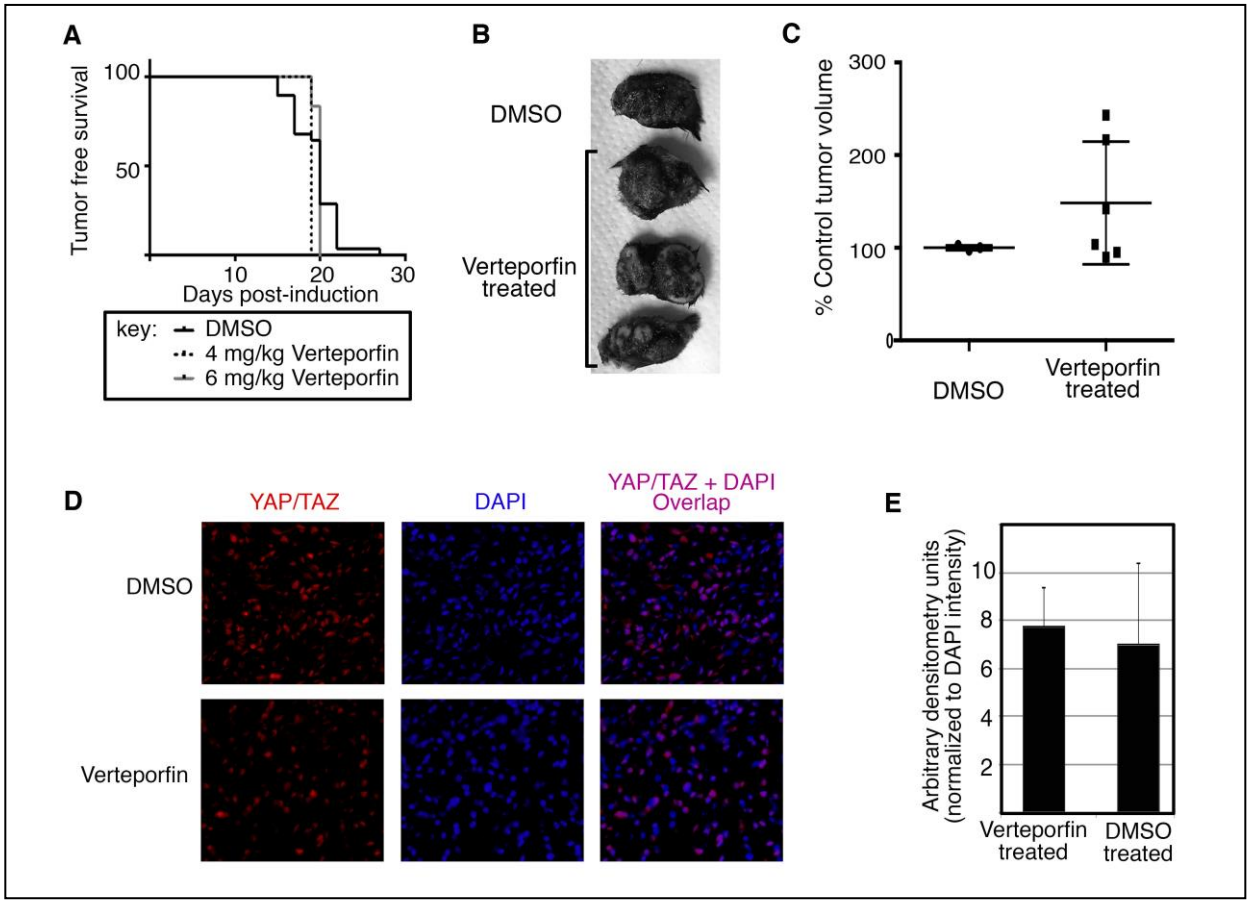


Figure 3.8. Verteporfin treatment does not inhibit tumor initiation or YAP/TAZ protein levels in murine melanoma model. Topical administration of 1-2 µl of 1.9 mg/ml (5mM) 4 - hydroxytamoxifen (4HT) was applied on three consecutive days to 12 week old *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ mice. The mice were subjected to 4 and 6 mg/kg Verteporfin intraperitoneal injections every other day for the course of the study starting the first day of melanoma induction. (A) Verteporfin treatment does not significantly alter initiation of melanocytic tumors from DMSO control treated mice. Mice were observed daily for the presence of pigmented lesions at the location of tamoxifen treatment on shaved backskin. Percent of mice lesion free are graphed by percentage (y axis) over a time course (x axis). (B,C) Verteporfin treatment does not inhibit tumor progression of melanomas in the *Braf*^{CA}*; Tyr-CreERT2*; *Pten*$^{f/f}$ mice. Examples of

Figure 3.8 (continued) gross specimens post dissection are shown (B). Mouse tumors are measured with a caliper externally and volumes were calculated using the formula $V = a \times b^2/2$, where a is the largest diameter and b in the smallest (Carlsson, Gullberg, and Hafström 1983). Tumor sizes are normalized to percent of the average size for control (DMSO treated) tumors and graphed by group (C). There was not a significant difference in tumor size between treatment and control groups at 45 days post tumor induction ($p=0.262$). (D,E) Immunohistochemical stain for YAP/TAZ shows no reduction of YAP/TAZ levels in Verteporfin samples as compared to DMSO control. Tumor samples were fixed with formalin and subsequently paraffin embedded. 5 μM sections were cut and probed for YAP/TAZ. For the graph in (E), fluorescent staining for YAP/TAZ (red channel) was quantified by densitometry measurements using image analysis (ImageJ) from 3 samples and 10 separate images per sample, and the values were normalized to DAPI nuclear stain fluorescence (blue channel). There was not a significant difference in the fluorescent staining between Verteporfin treated and control DMSO treated groups ($p=0.326$).

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 OVERVIEW –

Since their initial discovery over 20 years ago, the transcriptional coactivators YAP and TAZ have emerged as crucial regulators of mammalian development and disease. More specifically, misregulation of these paralogous cofactors have been demonstrated to drive a variety of mammalian cancers in redundant fashions. My dissertation research focused on elucidating potential mechanisms that YAP and/or TAZ play in driving melanoma.

I first explored potential differences that YAP and TAZ have in driving melanoma progression and survival. I found that inhibition of YAP or inhibition of TAZ led to vastly different transcriptomes in melanoma (**Figure 2.4**), and that inhibition of YAP but not TAZ resulted in decreased melanoma cell invasion, growth, and focal adhesion numbers (**Figure 2.2, 2.3**). Furthermore, I found that YAP drove expression of ARPC5, a member of the 7 subunit actin modifying ARP2/3 complex (**Figure 2.5**). Direct inhibition of ARPC5 led to decreased focal adhesion numbers and migration in melanoma cells (**Figure 2.6**). Next, I examined whether inhibition of both YAP and TAZ through the use of small molecule inhibitor Verteporfin could reduce melanoma progression *in vivo*. My findings showed that while Verteporfin reduced melanoma migration (**Figure 3.2**), cell numbers (**Figure 3.2**), and both YAP and TAZ levels in cell culture (**Figure 3.1**), treatment in a transgenic melanoma murine model with a focus on clinically relevant drug levels did not inhibit tumor initiation, progression, or YAP/TAZ protein levels (**Figure 3.6**). Additionally, I found that Verteporfin treatment did not phenocopy dual YAP/TAZ knockdown (**Figure 3.4**). My findings are novel as they reveal stark differences in how YAP and TAZ drive melanoma, with YAP playing a predominantly larger role despite its

lower expression levels. Furthermore, my work on Verteporfin revealed that treatment with the small molecule inhibitor did not mirror direct YAP and TAZ inhibition as it resulted in significant differences in cellular morphology. This suggests off target effects of Verteporfin, limiting its therapeutic potential as a YAP and TAZ inhibitor.

In summary, these studies provide a foundation that YAP and TAZ have differential roles in melanoma, and that inhibition of YAP could provide a solid strategy for melanoma therapeutics. For the remainder of this last chapter, I will elaborate on potential future directions and how this could impact YAP/TAZ targeted therapeutics in melanoma.

4.2 MATERIALS AND METHODS

Immunoprecipitation –

To generate immunoprecipitation lysates for mass spec analysis, mel537 cells were sonicated in mPER lysis buffer (ThermoFisher Catalog #78501) supplemented with Halt Protease Inhibitor (ThermoFisher Catalog #78429). Immunoprecipitations with PureProteome Magnetic A/G beads using antibodies for YAP (Abcam catalog ab52771), TAZ (Sigma Aldrich Catalog # MABS1913), or normal human IgG (Sigma) were followed according to manufacturers' instructions for direct immunoprecipitations.

Tandem mass spectroscopy –

Immunoprecipitated lysates for YAP and TAZ were run on 3-15% Bis-Tris gels (Bio-Rad) and subsequently Coomassie stained (Biorad Catalog # 1610436) according to manufactures' instructions to ensure quality and quantity of immunoprecitated lysates. The gels

were then destained (Biorad Catalog #1610438) and excised into two sections per immunoprecipitated group, with each section corresponding to either 25-60 kd or 60-160 kd sizes. The resulting gel slices were submitted to Harvard Medical School's Taplin Mass Spectrocopy Facility (https://taplin.med.harvard.edu/home) for analysis.

4.3 How does YAP drive ARPC5 expression in melanoma?

In chapter 2, I explored downstream transcriptomic differences between YAP and TAZ in melanoma. RNA-sequencing revealed that ARP2/3 complex member ARPC5 is downregulated with YAP, but not TAZ, inhibition (**Figure 2.4**). I found this YAP specific downregulation of ARPC5 expression to be recapitulated in a panel of melanoma cells (**Figure 2.5**). However, it is unclear exactly how YAP drives expression of ARPC5. Does YAP drive expression of ARPC5 directly or indirectly? Here, we will explore this possibility in depth.

YAP and TAZ have been demonstrated to drive oncogenic growth by mostly acting through distal enhancers, located greater than 100,000 base pairs away from the target promoter, that form chromatin loops to induce contact and gene expression of the enhancers with the target promoters and genes respectively (Zanconato et al. 2015). In liver cancer cells, YAP drives oncogenic growth through binding to TEAD and subsequent recruitment of the Mediator complex through the MED12 subunit to drive transcriptional elongation (Galli et al. 2015). The mediator complex consists of ~30 subunits that drive various cell processes, including cancer cell proliferation and progression, by inducing transcription of downstream targets through associations with transcription factors, chromatin regulators, and RNA polymerase II (Schiano et al. 2014; Yin and Wang 2014) . While the authors hypothesize that YAP and TAZ both contain

the ability to link distal enhancers to the Mediator complex to drive transcription, TAZ is not directly investigated. This suggest several mechanisms by which YAP could function differently from TAZ in our melanoma cells: (1) While YAP and TAZ can both bind TEAD, only YAP is able to bind the Mediator complex at a distal enhancer to directly drive expression of ARPC5; (2) YAP, but not TAZ, can bind an unknown transcription factor at a distal enhancer to directly drive expression of ARPC5 through the Mediator complex; (3) YAP does not directly regulate expression of ARPC5, but rather controls expression through another YAP specific factor that directly regulates ARPC5 expression.

The first mechanism is that only YAP contains the ability to bind the Mediator complex in melanoma. YAP would bind to TEAD which then binds to a distal enhancer of ARPC5. YAP interaction with the Mediator complex via the MED12 subunit subsequently recruits RNA pol II to promote ARPC5 expression. One potential caveat to this hypothesis is that TAZ was demonstrated to interact with MED15, another member of the mediator complex, in HEK-293T cells (Varelas et al. 2008). While it is a large 30 subunit complex, the Mediator complex is divided into 4 main modules (head, tail, middle, and CDK8 kinase) where MED15 is a subunit of the tail module and MED12 a subunit of the CDK8 kinase module (Bourbon 2008; Tsai et al. 2014). The head/middle modules are implicated to act as the essential modules in driving transcriptional regulation, whereas the tail and CDK8 kinase modules act as regulatory modules in (Soutourina 2018). Furthermore, the composition of the modules is variable, allowing for functional flexibility (Poss, Ebmeier, and Taatjes 2013; Allen and Taatjes 2015). Therefore, while YAP and TAZ have been shown to bind to Mediator complex subunits in different cell settings, it is still possible that YAP exclusively binds the Mediator complex in melanoma.

The second mechanism is that YAP interacts with an unknown transcription factor besides TEAD that TAZ cannot bind. YAP could bind another transcription factor through a YAP specific domain, which in turn causes YAP to recruit the mediator complex to the distal enhancer to drive ARPC5 expression. While YAP and TAZ share over 60% similarities, YAP possesses unique regions that are not found in TAZ, such as an SH3 binding domain and a proline rich peptide. As TAZ could not bind the unknown transcription factor, only YAP mediated recruitment to the distal promoter could drive ARPC5 expression.

To examine which of the two hypotheses above is correct, we would first perform Hi-C analysis to specifically identify distal enhancers of the ARPC5 transcriptional start site. Subsequent ChIP analysis for YAP and MED12 would indicate which enhancers contain both YAP and MED12 interaction. Sequence analysis for the distal enhancers could determine if any of the enhancers have TEAD binding sites, and luciferase assays comparing those enhancer sites with wild-type or mutant TEAD binding elements would determine whether those TEAD binding sites were active. Lastly, we would perform separate co-immunoprecipitation assays with YAP and TAZ to determine their binding to MED12 or other mediator complex proteins in melanoma cells. If both YAP and TAZ are able to bind MED12 and there are no TEAD binding sites on the distal enhancers, this suggests that YAP must be binding another transcription factor to recruit the mediator complex. If only YAP is able to bind MED12 and there are active TEAD binding sites on the distal enhancers, then this suggests that YAP drives ARPC5 expression through unique binding to the mediator complex. If the enhancers contain TEAD binding sites and both YAP/TAZ bind the mediator complex, then it is possible that there are additional binding sites for non-TEAD transcription factors on the distal enhancers. To investigate this

possibility, we would identify potential YAP binding partners by enriching for nuclei in melanoma cells followed by separate immunoprecipitation assays for YAP and TAZ, and subsequent tandem mass spectrometry (MS/MS) analysis. Any transcription factors that are unique to the YAP, but not TAZ, MS/MS group would be a potential candidate for YAP to bind with to drive ARPC5 through the distal enhancer. Analysis of the DNA binding elements for the MS/MS discovered YAP transcription factors would show whether or not those elements are on the distal enhancer. If they are, those transcription factors could all be driving ARPC5 expression. Subsequent knockdowns for those transcription factors and an examination of ARPC5 expression would reveal whether or not those candidates are upstream of ARPC5.

Lastly, the third possible situation is that YAP controls expression of ARPC5 indirectly. One way to initially examine this would be to search for known regulators of ARPC5 in our YAP knockdown RNA-sequencing results. Regardless of which theory holds true, the results would provide novel mechanisms for YAP specific regulation of ARPC5 in melanoma.

4.4 Do other YAP specific targets play major roles in melanoma progression and/or survival?

The results of the RNA-sequencing in chapter 2 (**Figure 2.4**) demonstrated that YAP and TAZ regulate different transcriptomes in melanoma, and that there is generally very little overlap between them. In chapter 2, I took an in-depth examination of the YAP specific target ARPC5. However, ARPC5 is only 1 of the 264 YAP specific genes from the RNA-sequencing experiment (**Figure 2.4**). Furthermore, pathway analysis of the 264 genes revealed high enrichment of genes involved in cell growth and proliferation (**Figure 2.4**). As YAP inhibition

resulted in decreased melanoma cell numbers and direct ARPC5 inhibition did not alter melanoma cell numbers, it is reasonable to postulate that other YAP specific genes are regulating cell growth and proliferation. Here, we explore the hypothesis that the genes BIRC5 and DUSP, both of whose expression is altered only with YAP, but not TAZ, inhibition, is controlling melanoma progression and survival.

In other cancers, BIRC5 and DUSP5 have already been implicated in driving several aspects of carcinoma progression, including cancer cell growth (Dual Specificity Phosphatase 5 - DUSP5) and survival (Baculoviral IAP Repeat Containing 5 - BIRC5) (X. Y. Wang et al. 2020; Fiziev et al. 2017; Dai et al. 2020; Wu et al. 2020). While our results indicate that these genes are downregulated in melanoma after inhibition of YAP, we have yet to validate them in other melanoma cell lines. To examine this possibility, we would first knockdown YAP, TAZ, and YAP/TAZ in a panel of human melanoma cell lines to examine whether BIRC5 and/or DUSP5 are similarly downregulated in a YAP specific manner. Once we have determined whether BIRC5, DUSP5, or both BIRC5/DUSP5 are downregulated across a panel of melanoma lines, we would take a gene specific approach to investigate the ability of these genes to control melanoma cell numbers.

Baculoviral IAP repeat-containing protein 5 (BIRC5) acts as an inhibitor of apoptosis. YAP inhibition results in a decrease in melanoma cell numbers. Thus, it is possible that downregulation of BIRC5 is responsible for the decreased cell number phenotype. First, I would need to directly inhibit BIRC5 to investigate whether the decrease in cell number from YAP is through downregulation of BIRC5. If cell numbers are decreased with direct inhibition of BIRC5, I would then assay for apoptosis through a measurement of caspase 3/7 activity. If direct

inhibition of BIRC5 and direct inhibition of YAP both led to an increase in apoptosis, I would then attempt to rescue the decreased cell number phenotype in our YAP inhibited cells with exogeneous expression of BIRC5 to conclusively demonstrate that YAP controls melanoma cell numbers through regulation of BIRC5. Being the fourth most upregulated mRNA in human cancers, BIRC5 serves as a potentially attractive target in cancer therapeutics (Velculescu et al. 1999; Wheatley and Altieri 2019). Despite the extensive studies into BIRC5 regulation and function since its discovery over 20 years ago, there are no known BIRC5 targeted cancer therapies in the clinic (Li, Aljahdali, and Ling 2019; Wheatley and Altieri 2019). Thus, it would be interesting to investigate its potential regulation by YAP in melanoma, providing a potential therapeutic avenue to target BIRC5 indirectly.

One function of Dual specificity protein phosphatase 5 (DUSP5) is to negatively regulate the MAPK pathway through its interaction with the ERK1/2 MAP kinases (Buffet et al. 2015). The Mitogen Activated Protein Kinase (MAPK) pathway has been shown to play key roles in melanoma growth and progression. In fact, ~50% and ~15% of all melanoma patients carry a mutation to two key MAPK pathway members, BRAF and NRAS respectively (Davies et al. 2002). Both mutations are activating mutations that drive melanoma cell growth and proliferation. Furthermore, activation of YAP has been implicated in helping melanoma cells gain BRAF inhibitor resistance (Fisher et al. 2017; M. H. Kim et al. 2016b). As DUSP5 is able to negatively regulate the MAPK pathway by acting as a phosphatase for MAPK pathway members, we hypothesize that YAP can drive melanoma proliferation through its negative regulation of DUSP5 (C. Y. Huang and Tan 2012; Kucharska et al. 2009). To test this hypothesis, I would first assay for MAPK activity in YAP inhibited melanoma cells by testing

for an increase in ERK1/2 phosphorylation. If MAPK activity is reduced, I would then directly inhibit DUSP5 to see if I get similar results to YAP inhibition. An additional rescue experiment for MAPK activity with endogenous expression of DUSP5 in YAP inhibited cells would provide further proof that YAP is exerting its effect on the MAPK pathway through DUSP5. Lastly, I would examine whether cell growth is increased using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assays in both DUSP5 and YAP inhibited cells. If the experiment results in decreased cell growth due to inhibition of DUSP5 and ERK1/2 phosphorylation is increased, this would suggest that YAP helps to drive melanoma cell proliferation via the MAPK pathway through negative regulation of DUSP5. Similar to BIRC5, elucidation of this potential pathway could provide a therapeutic avenue for inhibition of melanoma growth through targeting YAP.

While it is intriguing to hypothesize that either DUSP5 or BIRC5 is the sole regulator in controlling cell numbers, it is more likely that YAP controls melanoma cell numbers through the regulation of both BIRC5 and DUSP5, as well as others not discussed here. It will be interesting to examine to what extent YAP regulated targets are able to drive melanoma cell growth and how many YAP unique genes are involved in this process.

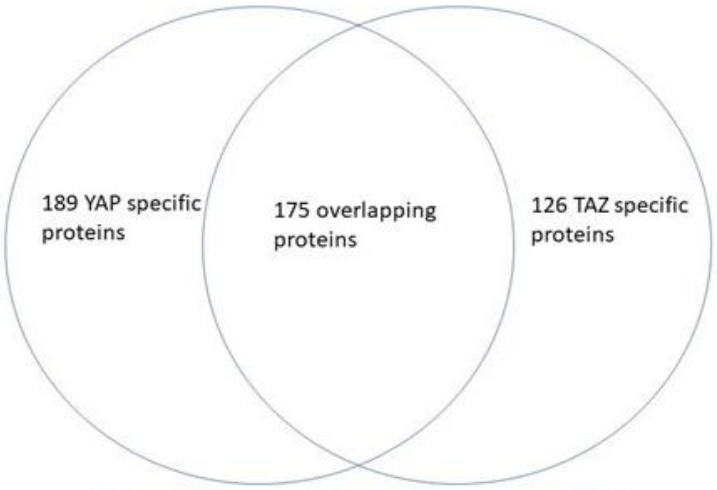
4.5 Does YAP differentially regulate melanoma through YAP specific binding partners?

While my investigation into YAP and TAZ differences have demonstrated that the two cofactors have differential functions and transcriptomes in melanoma, how they are able to have differing functions is not addressed. In chapter 1, I reviewed overlapping and unique domains that YAP and TAZ contain. As YAP and TAZ exert many of their downstream transcriptional

effects and are regulated through interactions with other transcription factors, it is likely that the two transcriptional coregulators are able to differentially regulate melanoma through their unique protein binding domains. I performed Mass Spectrometry analysis on separate YAP and TAZ immunoprecipitated lysates to reveal unique YAP and TAZ binding partners. While there are a large number of overlapping interacting partners between the two cofactors, there are an even larger amount of YAP specific proteins (**Figure 4.1A**). This could be due to YAP containing larger numbers of unique protein domains and motifs. As detailed in chapter 2, I found that melanoma cell morphology and focal adhesion numbers were altered in a YAP specific manner. A closer examination of the MS/MS results revealed 13 actin regulating and 8 tubulin binding proteins respectively. Furthermore, 5 out of the 13 actin binding proteins have SH3 domains (**Figure 4.1B**). Taking into account that YAP, but not TAZ, contains a SH3 binding protein domain, these proteins could be novel binding partners with YAP. As described in chapter 1, YAP can both regulate the actin cytoskeleton, as well as be regulated by the actin cytoskeleton. While YAP activity is usually described in a transcriptional manner, there is evidence that YAP can regulate cellular functions through non-transcriptional mechanisms. In *D. melanogaster* development, Yki promotes myosin activation at the cell cortex in to increase cellular tension and subsequent Yki activation independent of its transcriptional activity (J. Xu et al. 2018). Furthermore, cytoplasmic YAP and TAZ can inhibit TGF-β-SMAD signaling (Varelas, Samavarchi-Tehrani, et al. 2010). Thus, it is plausible that any of the five actin modifiers could be regulated by YAP or function in regulating YAP localization. To examine this theory, I would first perform co-immunoprecipitation experiments for YAP and any of the actin modifiers containing the SH3 binding domains to determine if MS/MS results can be recapitulated in other

cell lines. If any of the actin modifiers pulldown with YAP, I could next determine whether this binding is through the SH3 binding element on YAP by exogenously expressing a GFP-YAP Δ SH3 and performing immunoprecipitation assays for GFP and probing for the specific actin modifier. If the SH3 domain is not responsible for YAP is binding to these actin modifiers, we could perform deletion analysis of the YAP protein until the YAP-actin modifier interactions are lost to determine which domains are responsible for the interaction. Additionally, a comparison of GFP-YAP localization to GFP-YAP Δ SH3 would help to determine where these interactions could be exerting their effects in YAP melanoma biology. Lastly, attempting to rescue the YAP phenotypes with expression of YAP Δ SH3, or whichever domain was shown to be crucial for the YAP-actin modifier interaction, would determine which of the YAP phenotypes are regulated by their theoretical binding. These findings could potentially reveal novel ways that YAP, but not TAZ, exerts its effect on the actin cytoskeleton. Conversely, it could also reveal novel ways that YAP is regulated by the actin cytoskeleton. Regardless, elucidation of these YAP novel interactions will help to provide further clarity into how YAP uniquely drives melanoma.

A



B

Actin	Tubulin
CASK	IFT81
PLCG1	SKA3
ASAP1	NME7
NCKIPSD	PPP4C
SORBS2	DCLK1
ACTL6A	CLIP2
CNN1	TUBB6
MKL2	TPPP
AFAP1	
ABLIM3	
SSFA2	
ACTR2	
DIAPH1	

Figure 4.1: Mass Spectrometry analysis reveals unique YAP and TAZ binding partners with SH3 binding domains. (A) Venn Diagram schematic representing the total numbers of unique protein hits from tandem mass spectroscopy analysis of separate YAP and TAZ immunoprecipitations. (B) List of cytoskeleton binding or regulating proteins from the YAP specific proteins group. Highlighted in yellow are proteins that contain SH3 domains.

4.6 Are YAP and TAZ viable targets for melanoma therapeutics?

In Chapter 1, I reviewed several current therapies for melanoma. While many transcription factors have been demonstrated to play active roles in driving melanoma progression and survival, transcription factors have been historically hard to target (Bushweller 2019). One way around this problem is to target key effector genes. This makes the transcriptional cofactors YAP and TAZ suitable therapeutic targets. Small molecule inhibitor Verteporfin disrupts YAP and TAZ interaction with TEAD proteins *in vitro* (Liu-Chittenden et al. 2012). Therefore, we examined the effect of Verteporfin on melanoma cells and a melanoma mouse model. Early results were promising, as Verteporfin treatment resulted in reduced melanoma migration (**Figure 3.2**), cell numbers (**Figure 3.2**), and both YAP and TAZ levels in cell culture (**Figure 3.1**). While others had shown decreased tumor growth in orthotopic mouse models, we wanted to simulate clinical conditions as closely as possible. But through the use of a transgenic melanoma mouse model ($Braf^{CA}$; Tyr-CreERT2; $Pten^{f/f}$), I found that treatment at clinically relevant levels with Verteporfin did not reduce melanoma initiation of progression (**Figure 3.6**). While my studies revealed that Verteporfin did not inhibit melanoma progression or initiation under our conditions, it does not conclusively prove that YAP and/or TAZ are not viable therapeutic targets. As dual YAP/TAZ knockdown did not phenocopy Verteporfin treatment, it is possible that Verteporfin has off target effects. Targeted therapies are becoming more and more prevalent in modern medicine. As such, it is imperative that newer generation therapeutic avenues limit off target properties which could have unwanted side effects for the patient. Aside from their described roles in cancer, YAP and TAZ are implicated in playing

redundant roles in stem cell regulation, wound repair, and normal tissue homeostasis (Varelas 2014; Moroishi et al. 2015; M. J. Lee et al. 2014; D. Han et al. 2015; Elbediwy et al. 2016). YAP specific therapies could inhibit YAP specific functions driving melanoma but allow TAZ to compensate for any essential functions in normal tissues. Taking into account my experimental results from Chapter 2, it is plausible that targeting YAP alone is a more robust plan in designing melanoma therapies.

4.7 Concluding Remarks

In my dissertation research, I elucidated functional differences between YAP and TAZ in human melanoma. I also identified a novel YAP unique downstream protein in ARPC5 that regulates melanoma cell migration and focal adhesion numbers in melanoma. Furthermore, I have also shown that Verteporfin, a small molecule inhibitor demonstrated to disrupt YAP/TAZ-TEAD interaction *in vitro*, does not appear efficacious in inhibiting melanoma progression and survival in a murine melanoma model. Taken together, these studies provide new insights into how YAP and TAZ differ in melanoma. YAP is shown to play a larger role in melanoma growth and survival than TAZ. As such, targeting just YAP, as opposed to both YAP and TAZ, would provide more benefit to the patient as it could potentially limit any unwanted side effects that inhibition of TAZ could have in the patient. It will be beneficial to elucidate other potential differences between YAP and TAZ in their regulation of melanoma and other cancers to provide a sturdier foundation for designing YAP and/or TAZ targeted therapies.

REFERENCES

Abella, Jasmine V.G., Chiara Galloni, Julien Pernier, David J. Barry, Svend Kjær, Marie France Carlier, and Michael Way. 2016. "Isoform Diversity in the Arp2/3 Complex Determines Actin Filament Dynamics." *Nature Cell Biology* 18 (1): 76–86. https://doi.org/10.1038/ncb3286.

Acerbi, I., L. Cassereau, I. Dean, Q. Shi, A. Au, C. Park, Y. Y. Chen, J. Liphardt, E. S. Hwang, and V. M. Weaver. 2015. "Human Breast Cancer Invasion and Aggression Correlates with ECM Stiffening and Immune Cell Infiltration." *Integrative Biology (United Kingdom)*. https://doi.org/10.1039/c5ib00040h.

Allen, Benjamin L., and Dylan J. Taatjes. 2015. "The Mediator Complex: A Central Integrator of Transcription." *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm3951.

American Cancer Society. 2019. "Facts & Figures 2019." *American Cancer Society*.

Aragón, Eric, Nina Goerner, Qiaoran Xi, Tiago Gomes, Sheng Gao, Joan Massagué, and Maria J. MacIas. 2012. "Structural Basis for the Versatile Interactions of Smad7 with Regulator WW Domains in TGF-β Pathways." *Structure*. https://doi.org/10.1016/j.str.2012.07.014.

Aragón, Eric, Nina Goerner, Alexia Ileana Zaromytidou, Qiaoran Xi, Albert Escobedo, Joan Massagué, and Maria J. Macias. 2011. "A Smad Action Turnover Switch Operated by WW Domain Readers of a Phosphoserine Code." *Genes and Development*. https://doi.org/10.1101/gad.2060811.

Arakaki, Aleena K.S., Wen An Pan, and Jo Ann Trejo. 2018. "GPCRs in Cancer: Protease-Activated Receptors, Endocytic Adaptors and Signaling." *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms19071886.

Ascierto, Paolo A., Georgina V. Long, Caroline Robert, Benjamin Brady, Caroline Dutriaux, Anna Maria Di Giacomo, Laurent Mortier, et al. 2019. "Survival Outcomes in Patients with Previously Untreated BRAF Wild-Type Advanced Melanoma Treated with Nivolumab Therapy: Three-Year Follow-up of a Randomized Phase 3 Trial." *JAMA Oncology*. https://doi.org/10.1001/jamaoncol.2018.4514.

Azzolin, Luca, Tito Panciera, Sandra Soligo, Elena Enzo, Silvio Bicciato, Sirio Dupont, Silvia Bresolin, et al. 2014. "YAP/TAZ Incorporation in the β-Catenin Destruction Complex Orchestrates the Wnt Response." *Cell*. https://doi.org/10.1016/j.cell.2014.06.013.

Becher, Oren J., and Eric C. Holland. 2006. "Genetically Engineered Models Have Advantages over Xenografts for Preclinical Studies." *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-05-3827.

Bertolotto, Corine, Fabienne Lesueur, Sandy Giuliano, Thomas Strub, Mahaut De Lichy, Karine Bille, Philippe Dessen, et al. 2011. "A SUMOylation-Defective MITF Germline Mutation Predisposes to Melanoma and Renal Carcinoma." *Nature*. https://doi.org/10.1038/nature10539.

Bhattacharya, Debadrita, Ana Paula Azambuja, and Marcos Simoes-Costa. 2020. "Metabolic Reprogramming Promotes Neural Crest Migration via Yap/Tead Signaling." *Developmental Cell*. https://doi.org/10.1016/j.devcel.2020.03.005.

Bork, Peer, and Marius Sudol. 1994. "The WW Domain: A Signalling Site in Dystrophin?" *Trends in Biochemical Sciences*. https://doi.org/10.1016/0968-0004(94)90053-1.

Bourbon, Henri Marc. 2008. "Comparative Genomics Supports a Deep Evolutionary Origin for the Large, Four-Module Transcriptional Mediator Complex." *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkn349.

Bressler, Neil M. 1999. "Photodynamic Therapy of Subfoveal Choroidal Neovascularization in Age- Related Macular Degeneration with Verteporfin: One-Year Results of 2 Randomized Clinical Trials - TAP Report 1." *Archives of Ophthalmology*. https://doi.org/10.1001/archopht.117.10.1329.

Bressler, Neil M., and Susan B. Bressler. 2000. "Photodynamic Therapy with Verteporfin (Visudyne): Impact on Ophthalmology and Visual Sciences." *Investigative Ophthalmology and Visual Science*.

Buffet, Camille, Maria Grazia Catelli, Karine Hecale-Perlemoine, Léopoldine Bricaire, Camille Garcia, Anne Gallet-Dierick, Stéphanie Rodriguez, Françoise Cormier, and Lionel Groussin. 2015. "Dual Specificity Phosphatase 5, a Specific Negative Regulator of ERK Signaling, Is Induced by Serum Response Factor and Elk-1 Transcription Factor." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0145484.

Busetti, A., M. Soncin, G. Jori, and M. A.J. Rodgers. 1999. "High Efficiency of Benzoporphyrin Derivative in the Photodynamic Therapy of Pigmented Malignant Melanoma." *British Journal of Cancer*. https://doi.org/10.1038/sj.bjc.6690131.

Bushweller, John H. 2019. "Targeting Transcription Factors in Cancer — from Undruggable to Reality." *Nature Reviews Cancer*. https://doi.org/10.1038/s41568-019-0196-7.

Cai, Hui, and Yan Xu. 2013. "The Role of LPA and YAP Signaling in Long-Term Migration of Human Ovarian Cancer Cells." *Cell Communication and Signaling*. https://doi.org/10.1186/1478-811X-11-31.

Calvo, Fernando, Nil Ege, Araceli Grande-Garcia, Steven Hooper, Robert P. Jenkins, Shahid I. Chaudhry, Kevin Harrington, et al. 2013. “Mechanotransduction and YAP-Dependent Matrix Remodelling Is Required for the Generation and Maintenance of Cancer-Associated Fibroblasts.” *Nature Cell Biology*. https://doi.org/10.1038/ncb2756.

Carlsson, G., B. Gullberg, and L. Hafström. 1983. “Estimation of Liver Tumor Volume Using Different Formulas-An Experimental Study in Rats.” *Journal of Cancer Research and Clinical Oncology*. https://doi.org/10.1007/BF00391826.

Chakraborty, Sayan, Kizito Njah, Ajaybabu V. Pobbati, Ying Bena Lim, Anandhkumar Raju, Manikandan Lakshmanan, Vinay Tergaonkar, Chwee Teck Lim, and Wanjin Hong. 2017. “Agrin as a Mechanotransduction Signal Regulating YAP through the Hippo Pathway.” *Cell Reports*. https://doi.org/10.1016/j.celrep.2017.02.041.

Chen, Henry I., and Marius Sudol. 1995. “The WW Domain of Yes-Associated Protein Binds a Proline-Rich Ligand That Differs from the Consensus Established for Src Homology 3-Binding Modules.” *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.92.17.7819.

Cheng, Jung Chien, Evan Y. Wang, Yuyin Yi, Avinash Thakur, Shu Huei Tsai, and Pamela A. Hoodless. 2018. “S1P Stimulates Proliferation by Upregulating CTGF Expression through S1PR2-Mediated YAP Activation.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-17-0681.

Choe, Min Ho, Yina Yoon, Joon Kim, Sang-Gu Hwang, Young-Hoon Han, and Jae-Sung Kim. 2018. “MiR-550a-3-5p Acts as a Tumor Suppressor and Reverses BRAF Inhibitor Resistance through the Direct Targeting of YAP.” *Cell Death & Disease* 9 (6): 640. https://doi.org/10.1038/s41419-018-0698-3.

Chua, Vivian, Dominic Lapadula, Clinita Randolph, Jeffrey L. Benovic, Philip B. Wedegaertner, and Andrew E. Aplin. 2017. “Dysregulated GPCR Signaling and Therapeutic Options in Uveal Melanoma.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-17-0007.

Cichorek, Mirosława, Małgorzata Wachulska, Aneta Stasiewicz, and Agata Tymińska. 2013. “Skin Melanocytes: Biology and Development.” *Postepy Dermatologii i Alergologii*. https://doi.org/10.5114/pdia.2013.33376.

Comin-Anduix, Begoña, Thinle Chodon, Hooman Sazegar, Douglas Matsunaga, Stephen Mock, Jason Jalil, Helena Escuin-Ordinas, Bartosz Chmielowski, Richard C. Koya, and Antoni Ribas. 2010. “The Oncogenic BRAF Kinase Inhibitor PLX4032/RG7204 Does Not Affect the Viability or Function of Human Lymphocytes across a Wide Range of Concentrations.” *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-10-1911.

Cooper, Cynthia D., and David W. Raible. 2009. “Mechanisms for Reaching the Differentiated State: Insights from Neural Crest-Derived Melanocytes.” *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2008.09.008.

Crowson, A. Neil, Cynthia Magro, Arlo Miller, and Martin C. Mihm. 2007. “The Molecular Basis of Melanomagenesis and the Metastatic Phenotype.” *Seminars in Oncology*. https://doi.org/10.1053/j.seminoncol.2007.09.007.

Dai, Jian Bo, Bei Zhu, Wei Jia Lin, Hai Yan Gao, Hong Dai, Lin Zheng, Wei Hai Shi, and Wei Xian Chen. 2020. “Identification of Prognostic Significance of BIRC5 in Breast Cancer Using Integrative Bioinformatics Analysis.” *Bioscience Reports*. https://doi.org/10.1042/BSR20193678.

Dankort, David, David P. Curley, Robert A. Cartlidge, Betsy Nelson, Anthony N. Karnezis, William E. Damsky, Mingjian J. You, Ronald A. DePinho, Martin McMahon, and Marcus Bosenberg. 2009. “BrafV600E Cooperates with Pten Loss to Induce Metastatic Melanoma.” *Nature Genetics*. https://doi.org/10.1038/ng.356.

Dasari, Venkata Ramesh, Virginia Mazack, Wen Feng, John Nash, David J. Carey, and Radhika Gogoi. 2017. “Verteporfin Exhibits YAP-Independent Anti-Proliferative and Cytotoxic Effects in Endometrial Cancer Cells.” *Oncotarget*. https://doi.org/10.18632/oncotarget.15614.

Davies, Helen, Graham R. Bignell, Charles Cox, Philip Stephens, Sarah Edkins, Sheila Clegg, Jon Teague, et al. 2002. “Mutations of the BRAF Gene in Human Cancer.” *Nature*. https://doi.org/10.1038/nature00766.

Day, Chi Ping, Glenn Merlino, and Terry Van Dyke. 2015. “Preclinical Mouse Cancer Models: A Maze of Opportunities and Challenges.” *Cell*. https://doi.org/10.1016/j.cell.2015.08.068.

Deng, Xiaochong, and Lin Fang. 2018. “VGLL4 Is a Transcriptional Cofactor Acting as a Novel Tumor Suppressor via Interacting with TEADs.” *American Journal of Cancer Research*.

Dethlefsen, Christine, Louise S. Hansen, Christian Lillelund, Christina Andersen, Julie Gehl, Jesper F. Christensen, Bente K. Pedersen, and Pernille Hojman. 2017. “Exercise-Induced Catecholamines Activate the Hippo Tumor Suppressor Pathway to Reduce Risks of Breast Cancer Development.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-16-3125.

Dobrokhotov, Oleg, Mikhail Samsonov, Masahiro Sokabe, and Hiroaki Hirata. 2018. “Mechanoregulation and Pathology of YAP/TAZ via Hippo and Non-Hippo Mechanisms.” *Clinical and Translational Medicine*. https://doi.org/10.1186/s40169-018-0202-9.

Dong, Jixin, Georg Feldmann, Jianbin Huang, Shian Wu, Nailing Zhang, Sarah A. Comerford, Mariana F F. Gayyed, Robert A. Anders, Anirban Maitra, and Duojia Pan. 2007. "Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals." *Cell*. https://doi.org/10.1016/j.cell.2007.07.019.

Dooley, Christopher M., Neha Wali, Ian M. Sealy, Richard J. White, Derek L. Stemple, John E. Collins, and Elisabeth M. Busch-Nentwich. 2019. "The Gene Regulatory Basis of Genetic Compensation during Neural Crest Induction." *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1008213.

Duchartre, Yann, Yong Mi Kim, and Michael Kahn. 2016. "The Wnt Signaling Pathway in Cancer." *Critical Reviews in Oncology/Hematology*. https://doi.org/10.1016/j.critrevonc.2015.12.005.

Dupont, Sirio. 2016. "Role of YAP/TAZ in Cell-Matrix Adhesion-Mediated Signalling and Mechanotransduction." *Experimental Cell Research*. https://doi.org/10.1016/j.yexcr.2015.10.034.

Dupont, Sirio, Leonardo Morsut, Mariaceleste Aragona, Elena Enzo, Stefano Giulitti, Michelangelo Cordenonsi, Francesca Zanconato, et al. 2011. "Role of YAP/TAZ in Mechanotransduction." *Nature*. https://doi.org/10.1038/nature10137.

Elbediwy, Ahmed, Zoé I. Vincent-Mistiaen, Bradley Spencer-Dene, Richard K. Stone, Stefan Boeing, Stefanie K. Wculek, Julia Cordero, et al. 2016. "Integrin Signalling Regulates YAP and TAZ to Control Skin Homeostasis." *Development (Cambridge)*. https://doi.org/10.1242/dev.133728.

Fedoroff, Oleg Y., Sharon A. Townson, Alexander P. Golovanov, Martin Baron, and Johanna M. Avis. 2004. "The Structure and Dynamics of Tandem WW Domains in a Negative Regulator of Notch Signaling, Suppressor of Deltex." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M404987200.

Feng, Xiaodong, Maria Sol Degese, Ramiro Iglesias-Bartolome, Jose P. Vaque, Alfredo A. Molinolo, Murilo Rodrigues, M. Raza Zaidi, et al. 2014. "Hippo-Independent Activation of YAP by the GNAQ Uveal Melanoma Oncogene through a Trio-Regulated Rho GTPase Signaling Circuitry." *Cancer Cell*. https://doi.org/10.1016/j.ccr.2014.04.016.

Ferrigno, Olivier, François Lallemand, Franck Verrecchia, Sébastien L'Hoste, Jacques Camonis, Azeddine Atfi, and Alain Mauviel. 2002. "Yes-Associated Protein (YAP65) Interacts with Smad7 and Potentiates Its Inhibitory Activity against TGF-Beta/Smad Signaling." *Oncogene* 21 (32): 4879–84. https://doi.org/10.1038/sj.onc.1205623.

Fisher, Matthew L., Daniel Grun, Gautam Adhikary, Wen Xu, and Richard L. Eckert. 2017.

"Inhibition of YAP Function Overcomes BRAF Inhibitor Resistance in Melanoma Cancer Stem Cells." *Oncotarget* 8 (66): 110257–72. https://doi.org/10.18632/oncotarget.22628.

Fiziev, Petko, Kadir C. Akdemir, John P. Miller, Emily Z. Keung, Neha S. Samant, Sneha Sharma, Christopher A. Natale, et al. 2017. "Systematic Epigenomic Analysis Reveals Chromatin States Associated with Melanoma Progression." *Cell Reports*. https://doi.org/10.1016/j.celrep.2017.03.078.

Freireich, E. J., E. A. Gehan, D. P. Rall, L. H. Schmidt, and H. E. Skipper. 1966. "Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey, and Man." *Cancer Chemotherapy Reports. Part 1*.

Galli, Giorgio G., Matteo Carrara, Wei Chien Yuan, Christian Valdes-Quezada, Basanta Gurung, Brian Pepe-Mooney, Tinghu Zhang, et al. 2015. "YAP Drives Growth by Controlling Transcriptional Pause Release from Dynamic Enhancers." *Molecular Cell*. https://doi.org/10.1016/j.molcel.2015.09.001.

Gandini, Sara, Francesco Sera, Maria Sofia Cattaruzza, Paolo Pasquini, Roberto Zanetti, Cinzia Masini, Peter Boyle, and Carmelo Francesco Melchi. 2005. "Meta-Analysis of Risk Factors for Cutaneous Melanoma: III. Family History, Actinic Damage and Phenotypic Factors." *European Journal of Cancer*. https://doi.org/10.1016/j.ejca.2005.03.034.

Ge, Liang, Dong Song Li, Fei Chen, Ji Dong Feng, Bai Li, and Tie Jun Wang. 2017. "TAZ Overexpression Is Associated with Epithelial-Mesenchymal Transition in Cisplatin-Resistant Gastric Cancer Cells." *International Journal of Oncology*. https://doi.org/10.3892/ijo.2017.3998.

Gee, Stephen T., Sharon L. Milgram, Kenneth L. Kramer, Frank L. Conlon, and Sally A. Moody. 2011. "Yes-Associated Protein 65 (YAP) Expands Neural Progenitors and Regulates Pax3 Expression in the Neural Plate Border Zone." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0020309.

Gibault, Floriane, Matthieu Corvaisier, Fabrice Bailly, Guillemette Huet, Patricia Melnyk, and Philippe Cotelle. 2016. "Non-Photoinduced Biological Properties of Verteporfin." *Current Medicinal Chemistry*. https://doi.org/10.2174/0929867323666160316125048.

Guo, Peng, Shengjie Kang, and Feifei Zhao. 2018. "High Expression of TAZ/YAP Promotes the Progression of Malignant Melanoma and Affects the Postoperative Survival of Patients." *Die Pharmazie* 73 (11): 662–65. https://doi.org/10.1691/ph.2018.8499.

Ha, Linan, Glenn Merlino, and Elena V. Sviaerskaya. 2008. "Melanomagenesis: Overcoming the Barrier of Melanocyte Senescence." *Cell Cycle*. https://doi.org/10.4161/cc.7.13.6230.

Halder, Georg, Sirio Dupont, and Stefano Piccolo. 2012. “Transduction of Mechanical and Cytoskeletal Cues by YAP and TAZ.” *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm3416.

Hall, Chad A., Runsheng Wang, Jiangyong Miao, Esther Oliva, Xiaoyun Shen, Thomas Wheeler, Susan G. Hilsenbeck, Sandra Orsulic, and Scott Goode. 2010. “Hippo Pathway Effector Yap Is an Ovarian Cancer Oncogene.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-10-1242.

Han, Dasol, Sung Hyun Byun, Soojeong Park, Juwan Kim, Inhee Kim, Soobong Ha, Mookwang Kwon, and Keejung Yoon. 2015. “YAP/TAZ Enhance Mammalian Embryonic Neural Stem Cell Characteristics in a Tead-Dependent Manner.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2015.01.077.

Han, Li Li, Xiao Ran Yin, and Shu Qun Zhang. 2018. “MiR-103 Promotes the Metastasis and EMT of Hepatocellular Carcinoma by Directly Inhibiting LATS2.” *International Journal of Oncology* 53 (6): 2433–44. https://doi.org/10.3892/ijo.2018.4580.

Hao, Yawei, Alex Chun, Kevin Cheung, Babak Rashidi, and Xiaolong Yang. 2008. “Tumor Suppressor LATS1 Is a Negative Regulator of Oncogene YAP.” *Journal of Biological Chemistry* 283 (9): 5496–5509. https://doi.org/10.1074/jbc.M709037200.

Harland, Mark, Mia Petljak, Carla Daniela Robles-Espinoza, Zhihao Ding, Nelleke A. Gruis, Remco van Doorn, Karen A. Pooley, et al. 2016. “Germline TERT Promoter Mutations Are Rare in Familial Melanoma.” *Familial Cancer*. https://doi.org/10.1007/s10689-015-9841-9.

Hindley, Christopher J., Alexandra Larisa Condurat, Vishal Menon, Ria Thomas, Luis M. Azmitia, Jason A. Davis, and Jan Pruszak. 2016. “The Hippo Pathway Member YAP Enhances Human Neural Crest Cell Fate and Migration.” *Scientific Reports*. https://doi.org/10.1038/srep23208.

Holden, Jeffrey K., and Christian N. Cunningham. 2018. “Targeting the Hippo Pathway and Cancer through the TEAD Family of Transcription Factors.” *Cancers*. https://doi.org/10.3390/cancers10030081.

Hong, Jeong Ho, Eun Sook Hwang, Michael T. McManus, Adam Amsterdam, Yu Tian, Ralitsa Kalmukova, Elisabetta Mueller, et al. 2005. “TAZ, a Transcriptional Modulator of Mesenchymal Stem Cell Differentiation.” *Science* 309 (5737): 1074–78. https://doi.org/10.1126/science.1110955.

Hong, Wanjin, and Kun Liang Guan. 2012. “The YAP and TAZ Transcription Co-Activators: Key Downstream Effectors of the Mammalian Hippo Pathway.” *Seminars in Cell and Developmental Biology*. Elsevier Ltd. https://doi.org/10.1016/j.semcdb.2012.05.004.

Hoock, Samira Catharina, Andreas Ritter, Kerstin Steinhäuser, Susanne Roth, Christian Behrends, Franz Oswald, Christine Solbach, Frank Louwen, Nina-Naomi Kreis, and Juping Yuan. 2019. “RITA Modulates Cell Migration and Invasion by Affecting Focal Adhesion Dynamics.” *Molecular Oncology* 13 (10): 2121–41. https://doi.org/10.1002/1878-0261.12551.

Hooijkaas, Anna I., Jules Gadiot, Martin Van Der Valk, Wolter J. Mooi, and Christian U. Blank. 2012. “Targeting BRAF V600E in an Inducible Murine Model of Melanoma.” *American Journal of Pathology*. https://doi.org/10.1016/j.ajpath.2012.06.002.

Horn, Susanne, Adina Figl, P. Sivaramakrishna Rachakonda, Christine Fischer, Antje Sucker, Andreas Gast, Stephanie Kadel, et al. 2013. “TERT Promoter Mutations in Familial and Sporadic Melanoma.” *Science*. https://doi.org/10.1126/science.1230062.

Hossain, Zakir, Safiah Mohamed Ali, Hui Ling Ko, Jianliang Xu, Chee Peng Ng, Ke Guo, Zeng Qi, Sathivel Ponniah, Wanjin Hong, and Walter Hunziker. 2007. “Glomerulocystic Kidney Disease in Mice with a Targeted Inactivation of Wwtr1.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (5): 1631–36. https://doi.org/10.1073/pnas.0605266104.

Hou, Yanhong, Viet Nhat Hung Le, Thomas Clahsen, Ann Charlott Schneider, Felix Bock, and Claus Cursiefen. 2017. “Photodynamic Therapy Leads to Time-Dependent Regression of Pathologic Corneal (Lymph) Angiogenesis and Promotes High-Risk Corneal Allograft Survival.” *Investigative Ophthalmology and Visual Science*. https://doi.org/10.1167/iovs.17-22904.

Howe, Louise R., and Anthony M.C. Brown. 2004. “Wht Signaling and Breast Cancer.” *Cancer Biology and Therapy*. https://doi.org/10.4161/cbt.3.1.561.

Howell, Michael, Christoph Borchers, and Sharon L Milgram. 2004. “Heterogeneous Nuclear Ribonuclear Protein U Associates with YAP and Regulates Its Co-Activation of Bax Transcription.” *The Journal of Biological Chemistry* 279 (25): 26300–306. https://doi.org/10.1074/jbc.M401070200.

Howlader N, Noone AM, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, et al. 2019. “SEER Cancer Statistics Review, 1975-2016.” *National Cancer Institute*.

Huang, Ching Yu, and Tse Hua Tan. 2012. “DUSPs, to MAP Kinases and Beyond.” *Cell and Bioscience*. https://doi.org/10.1186/2045-3701-2-24.

Huang, Jianbin, Shian Wu, Jose Barrera, Krista Matthews, and Duojia Pan. 2005a. “The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the Drosophila Homolog of YAP.” *Cell*. https://doi.org/10.1016/j.cell.2005.06.007.

Huang, Wei, Xianbo Lv, Chenying Liu, Zhengyu Zha, Heng Zhang, Ying Jiang, Yue Xiong, Qun Ying Lei, and Kun Liang Guan. 2012. “The N-Terminal Phosphodegron Targets TAZ/WWTR1 Protein for SCF β-TrCP-Dependent Degradation in Response to Phosphatidylinositol 3-Kinase Inhibition.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M112.382036.

Huang, Xiaojuan, Monique Beullens, Jiahai Zhang, Yi Zhou, Emilia Nicolaescu, Bart Lesage, Qi Hu, Jihui Wu, Mathieu Bollen, and Yunyu Shi. 2009. “Structure and Function of the Two Tandem WW Domains of the Pre-MRNA Splicing Factor FBP21 (Formin-Binding Protein 21).” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M109.024828.

Huggett, M. T., M. Jermyn, A. Gillams, R. Illing, S. Mosse, M. Novelli, E. Kent, et al. 2014. “Phase I/II Study of Verteporfin Photodynamic Therapy in Locally Advanced Pancreatic Cancer.” *British Journal of Cancer*. https://doi.org/10.1038/bjc.2014.95.

Hussussian, Christopher J., Jeffery P. Struewing, Alisa M. Goldstein, Paul A.T. Higgins, Delphine S. Ally, Michelle D. Sheahan, Wallace H. Clark, Margaret A. Tucker, and Nicholas C. Dracopoli. 1994. “Germline P16 Mutations in Familial Melanoma.” *Nature Genetics*. https://doi.org/10.1038/ng0994-15.

Jaalouk, Diana E., and Jan Lammerding. 2009. “Mechanotransduction Gone Awry.” *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm2597.

Jiang, N., K. Hjorth-Jensen, O. Hekmat, D. Iglesias-Gato, T. Kruse, C. Wang, W. Wei, et al. 2015. “In Vivo Quantitative Phosphoproteomic Profiling Identifies Novel Regulators of Castration-Resistant Prostate Cancer Growth.” *Oncogene*. https://doi.org/10.1038/onc.2014.206.

Jiao, Shi, Chuanchuan Li, Qian Hao, Haofei Miao, Lei Zhang, Lin Li, and Zhaocai Zhou. 2017. “VGLL4 Targets a TCF4-TEAD4 Complex to Coregulate Wnt and Hippo Signalling in Colorectal Cancer.” *Nature Communications*. https://doi.org/10.1038/ncomms14058.

Jiao, Shi, Huizhen Wang, Zhubing Shi, Aimei Dong, Wenjing Zhang, Xiaomin Song, Feng He, et al. 2014. “A Peptide Mimicking VGLL4 Function Acts as a YAP Antagonist Therapy against Gastric Cancer.” *Cancer Cell*. https://doi.org/10.1016/j.ccr.2014.01.010.

Kamb, A., D. Shattuck-Eidens, R. Eeles, Q. Liu, N. A. Gruis, W. Ding, C. Hussey, et al. 1994. “Analysis of the P16 Gene (CDKN2) as a Candidate for the Chromosome 9p Melanoma Susceptibility Locus.” *Nature Genetics*. https://doi.org/10.1038/ng0994-22.

Kanai, Fumihiko, Paola A. Marignani, Dilara Sarbassova, Ryohei Yagi, Randy A. Hall, Mark Donowitz, Akihiko Hisaminato, et al. 2000. “TAZ: A Novel Transcriptional Co-Activator

Regulated by Interactions with 14-3-3 and PDZ Domain Proteins." *EMBO Journal* 19 (24): 6778–91. https://doi.org/10.1093/emboj/19.24.6778.

Kanelis, Voula, Neil A. Farrow, Lewis E. Kay, Daniela Rotin, and Julie D. Forman-Kay. 1998. "NMR Studies of Tandem WW Domains of Nedd4 in Complex with a PY Motif-Containing Region of the Epithelial Sodium Channel." *Biochemistry and Cell Biology*. https://doi.org/10.1139/o98-042.

Kang, Jiaxin, Jian Wang, Zhuang Yao, Yuanzhao Hu, Shijie Ma, Qin Fan, Feng Gao, Yan Sun, and Jianwei Sun. 2018. "Fascin Induces Melanoma Tumorigenesis and Stemness through Regulating the Hippo Pathway." *Cell Communication and Signaling* 16 (1). https://doi.org/10.1186/s12964-018-0250-1.

Kang, Wei, Joanna H.M. Tong, Anthony W.H. Chan, Tin Lap Lee, Raymond W.M. Lung, Patrick P.S. Leung, Ken K.Y. So, et al. 2011. "Yes-Associated Protein 1 Exhibits Oncogenic Property in Gastric Cancer and Its Nuclear Accumulation Associates with Poor Prognosis." *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-10-2467.

Kasanov, Jeremy, Gregorio Pirozzi, Albert J. Uveges, and Brian K. Kay. 2001. "Characterizing Class I WW Domains Defines Key Specificity Determinants and Generates Mutant Domains with Novel Specificities." *Chemistry and Biology*. https://doi.org/10.1016/S1074-5521(01)00005-9.

Kennedy, Cornelis, Jeanet Ter Huurne, Marjo Berkhout, Nelleke Gruis, Maarten Bastiaens, W. Bergman, R. Willemze, and Jan Nico Bouwes Bavinck. 2001. "Melanocortin 1 Receptor (MC1R) Gene Variants Are Associated with an Increased Risk for Cutaneous Melanoma Which Is Largely Independent of Skin Type and Hair Color." *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.0022-202x.2001.01421.x.

Kim, Ji Eun, Graeme J. Finlay, and Bruce C. Baguley. 2013. "The Role of the Hippo Pathway in Melanocytes and Melanoma." *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2013.00123.

Kim, Min Hwan, Jongshin Kim, Hyowon Hong, Si-Hyung Lee, June-Koo Lee, Eunji Jung, and Joon Kim. 2016a. " Actin Remodeling Confers BRAF Inhibitor Resistance to Melanoma Cells through YAP / TAZ Activation ." *The EMBO Journal*. https://doi.org/10.15252/embj.201592081.

Kinoshita, Takashi, Nijiro Nohata, Haruko Watanabe-Takano, Hirofumi Yoshino, Hideo Hidaka, Lisa Fujimura, Miki Fuse, et al. 2012. "Actin-Related Protein 2/3 Complex Subunit 5 (ARPC5) Contributes to Cell Migration and Invasion and Is Directly Regulated by Tumor-Suppressive MicroRNA-133a in Head and Neck Squamous Cell Carcinoma." *International Journal of Oncology* 40 (6): 1770–78. https://doi.org/10.3892/ijo.2012.1390.

Kléber, Maurice, Hye Youn Lee, Heiko Wurdak, Johanna Buchstaller, Martin M. Riccomagno, Lars M. Ittner, Ueli Suter, Douglas J. Epstein, and Lukas Sommer. 2005. "Neural Crest Stem Cell Maintenance by Combinatorial Wnt and BMP Signaling." *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200411095.

Knecht, Anne K., and Marianne Bronner-Fraser. 2002. "Induction of the Neural Crest: A Multigene Process." *Nature Reviews Genetics*. https://doi.org/10.1038/nrg819.

Konsavage, Wesley M., Sydney L. Kyler, Sherri A. Rennoll, Ge Jin, and Gregory S. Yochum. 2012. "Wnt/β-Catenin Signaling Regulates Yes-Associated Protein (YAP) Gene Expression in Colorectal Carcinoma Cells." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M111.327767.

Kotelevets, Larissa, and Eric Chastre. 2020. "Rac1 Signaling: From Intestinal Homeostasis to Colorectal Cancer Metastasis." *Cancers*. https://doi.org/10.3390/cancers12030665.

Kozlowski, James M., Ian R. Hart, Isaiah J. Fidler, and Nabil Hanna. 1984. "A Human Melanoma Line Heterogeneous with Respect to Metastatic Capacity in Athymic Nude Mice." *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/72.4.913.

Kristal Kaan, Hung Yi, Siew Wee Chan, Siew Kim Joyce Tan, Fusheng Guo, Chun Jye Lim, Wanjin Hong, and Haiwei Song. 2017. "Crystal Structure of TAZ-TEAD Complex Reveals a Distinct Interaction Mode from That of YAP-TEAD Complex." *Scientific Reports*. https://doi.org/10.1038/s41598-017-02219-9.

Kubic, Jennifer D., Kacey P. Young, Rebecca S. Plummer, Anton E. Ludvik, and Deborah Lang. 2008. "Pigmentation PAX-Ways: The Role of Pax3 in Melanogenesis, Melanocyte Stem Cell Maintenance, and Disease." *Pigment Cell and Melanoma Research*. https://doi.org/10.1111/j.1755-148X.2008.00514.x.

Kucharska, Anna, Linda K. Rushworth, Christopher Staples, Nick A. Morrice, and Stephen M. Keyse. 2009. "Regulation of the Inducible Nuclear Dual-Specificity Phosphatase DUSP5 by ERK MAPK." *Cellular Signalling*. https://doi.org/10.1016/j.cellsig.2009.07.015.

Kumar, Deepak, Erez Nitzan, and Chaya Kalcheim. 2019. "YAP Promotes Neural Crest Emigration through Interactions with BMP and Wnt Activities." *Cell Communication and Signaling*. https://doi.org/10.1186/s12964-019-0383-x.

Kurochkina, Natalya, and Udayan Guha. 2013. "SH3 Domains: Modules of Protein-Protein Interactions." *Biophysical Reviews*. https://doi.org/10.1007/s12551-012-0081-z.

Kuzumaki, Naoko, Atsuo Suzuki, Michiko Narita, Takahiro Hosoya, Atsumi Nagasawa, Satoshi Imai, Kohei Yamamizu, et al. 2012. "Multiple Analyses of G-Protein Coupled Receptor

(GPCR) Expression in the Development of Gefitinib-Resistance in Transforming Non-Small-Cell Lung Cancer." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0044368.

Lamar, John M., Yuxuan Xiao, Emily Norton, Zhi Gang Jiang, Genevieve M. Gerhard, Simrin Kooner, Janine S.A. Warren, and Richard O. Hynes. 2019. "SRC Tyrosine Kinase Activates the YAP/TAZ Axis and Thereby Drives Tumor Growth and Metastasis." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.RA118.004364.

Lamar, John M, Patrick Stern, Hui Liu, Jeffrey W Schindler, Zhi-Gang Jiang, and Richard O Hynes. 2012. "The Hippo Pathway Target, YAP, Promotes Metastasis through Its TEAD-Interaction Domain." *Proceedings of the National Academy of Sciences of the United States of America* 109 (37): E2441-50. https://doi.org/10.1073/pnas.1212021109.

Lang, Deborah, Fabian Chen, Rita Milewski, Jun Li, Min Min Lu, and Jonathan A. Epstein. 2000. "Pax3 Is Required for Enteric Ganglia Formation and Functions with Sox10 to Modulate Expression of C-Ret." *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI10828.

Lang, Deborah, Min Min Lu, Ll Huang, Kurt A. Engleka, Maozhen Zhang, Emily Y. Chu, Shari Lipner, Arthur Skoultchi, Sarah E. Millar, and Jonathan A. Epstein. 2005. "Pax3 Functions at a Nodal Point in Melanocyte Stem Cell Differentiation." *Nature*. https://doi.org/10.1038/nature03292.

Lee, Ho Jin, and Jie J. Zheng. 2010. "PDZ Domains and Their Binding Partners: Structure, Specificity, and Modification." *Cell Communication and Signaling*. https://doi.org/10.1186/1478-811X-8-8.

Lee, Hyunkyoung, Jong Shu Kim, and Euikyung Kim. 2012. "Fucoidan from Seaweed Fucus Vesiculosus Inhibits Migration and Invasion of Human Lung Cancer Cell via PI3K-Akt-MTOR Pathways." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0050624.

Lee, Min Jung, Mi Ran Byun, Makoto Furutani-Seiki, Jeong Ho Hong, and Han Sung Jung. 2014. "YAP and TAZ Regulate Skin Wound Healing." *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2013.339.

Lee, Yoonmi, Nam Hee Kim, Eunae Sandra Cho, Ji Hye Yang, Yong Hoon Cha, Hee Eun Kang, Jun Seop Yun, et al. 2018. "Dishevelled Has a YAP Nuclear Export Function in a Tumor Suppressor Context-Dependent Manner." *Nature Communications*. https://doi.org/10.1038/s41467-018-04757-w.

Levy, Dan, Yaarit Adamovich, Nina Reuven, and Yosef Shaul. 2008. "Yap1 Phosphorylation by C-Abl Is a Critical Step in Selective Activation of Proapoptotic Genes in Response to DNA Damage." *Molecular Cell*. https://doi.org/10.1016/j.molcel.2007.12.022.

Li, Fengzhi, Ieman Aljahdali, and Xiang Ling. 2019. “Cancer Therapeutics Using Survivin BIRC5 as a Target: What Can We Do after over Two Decades of Study?” *Journal of Experimental and Clinical Cancer Research*. https://doi.org/10.1186/s13046-019-1362-1.

Liang, Chun Chi, Ann Y. Park, and Jun Lin Guan. 2007. “In Vitro Scratch Assay: A Convenient and Inexpensive Method for Analysis of Cell Migration in Vitro.” *Nature Protocols*. https://doi.org/10.1038/nprot.2007.30.

Liu-Chittenden, Yi, Bo Huang, Joong Sup Shim, Qian Chen, Se Jin Lee, Robert A. Anders, Jun O. Liu, and Duojia Pan. 2012. “Genetic and Pharmacological Disruption of the TEAD-YAP Complex Suppresses the Oncogenic Activity of YAP.” *Genes and Development*. https://doi.org/10.1101/gad.192856.112.

Liu, Chen Ying, Tong Yu, Yuji Huang, Long Cui, and Wanjin Hong. 2017. “ETS (E26 Transformation-Specific) up-Regulation of the Transcriptional Co-Activator TAZ Promotes Cell Migration and Metastasis in Prostate Cancer.” *Journal of Biological Chemistry* 292 (22): 9420–30. https://doi.org/10.1074/jbc.M117.783787.

Liu, Tianyi, Linli Zhou, Kun Yang, Kentaro Iwasawa, Ana Luisa Kadekaro, Takanori Takebe, Thomas Andl, and Yuhang Zhang. 2019. “The β-Catenin/YAP Signaling Axis Is a Key Regulator of Melanoma-Associated Fibroblasts.” *Signal Transduction and Targeted Therapy*. https://doi.org/10.1038/s41392-019-0100-7.

Low, Boon Chuan, Catherine Qiurong Pan, G. V. Shivashankar, Alexander Bershadsky, Marius Sudol, and Michael Sheetz. 2014. “YAP/TAZ as Mechanosensors and Mechanotransducers in Regulating Organ Size and Tumor Growth.” *FEBS Letters*. https://doi.org/10.1016/j.febslet.2014.04.012.

Lu, Yi, Tiantian Wu, Orit Gutman, Huasong Lu, Qiang Zhou, Yoav I. Henis, and Kunxin Luo. 2020. “Phase Separation of TAZ Compartmentalizes the Transcription Machinery to Promote Gene Expression.” *Nature Cell Biology*. https://doi.org/10.1038/s41556-020-0485-0.

Luo, Jiaqian, and Fa-Xing Yu. 2019. “GPCR-Hippo Signaling in Cancer.” *Cells*. https://doi.org/10.3390/cells8050426.

Lustig, B., and J. Behrens. 2003. “The Wnt Signaling Pathway and Its Role in Tumor Development.” *Journal of Cancer Research and Clinical Oncology*. https://doi.org/10.1007/s00432-003-0431-0.

Macias, Maria J., Marko Hyvönen, Elena Baraldi, Johan Schultz, Marius Sudol, Matti Saraste, and Hartmut Oschkinat. 1996. “Structure of the WW Domain of a Kinase-Associated Protein Complexed with a Proline-Rich Peptide.” *Nature*. https://doi.org/10.1038/382646a0.

Makita, Ryosuke, Yasunobu Uchijima, Koichi Nishiyama, Tomokazu Amano, Qin Chen, Takumi Takeuchi, Akihisa Mitani, et al. 2008. "Multiple Renal Cysts, Urinary Concentration Defects, and Pulmonary Emphysematous Changes in Mice Lacking TAZ." *American Journal of Physiology. Renal Physiology* 294 (3): F542-53. https://doi.org/10.1152/ajprenal.00201.2007.

Manderfield, Lauren J., Kurt A. Engleka, Haig Aghajanian, Mudit Gupta, Steven Yang, Li Li, Julie E. Baggs, John B. Hogenesch, Eric N. Olson, and Jonathan A. Epstein. 2014. "Pax3 and Hippo Signaling Coordinate Melanocyte Gene Expression in Neural Crest." *Cell Reports*. https://doi.org/10.1016/j.celrep.2014.10.061.

Martinez, Bridget, Yongchao Yang, Donald Mario Robert Harker, Charles Farrar, Harshini Mukundan, Pulak Nath, and David Mascareñas. 2019. "YAP/TAZ Related BioMechano Signal Transduction and Cancer Metastasis." *Frontiers in Cell and Developmental Biology*. Frontiers Media S.A. https://doi.org/10.3389/fcell.2019.00199.

McGill, Gaël G., Martin Horstmann, Hans R. Widlund, Jinyan Du, Gabriela Motyckova, Emi K. Nishimura, Yi Ling Lin, et al. 2002. "Bcl2 Regulation by the Melanocyte Master Regulator Mitf Modulates Lineage Survival and Melanoma Cell Viability." *Cell*. https://doi.org/10.1016/S0092-8674(02)00762-6.

Meng, Zhipeng, Toshiro Moroishi, Violaine Mottier-Pavie, Steven W. Plouffe, Carsten G. Hansen, Audrey W. Hong, Hyun Woo Park, et al. 2015. "MAP4K Family Kinases Act in Parallel to MST1/2 to Activate LATS1/2 in the Hippo Pathway." *Nature Communications* 6 (October). https://doi.org/10.1038/ncomms9357.

Menzel, Moritz, Diana Meckbach, Benjamin Weide, Nora C. Toussaint, Karin Schilbach, Seema Noor, Thomas Eigentler, et al. 2014a. "In Melanoma, Hippo Signaling Is Affected by Copy Number Alterations and YAP1 Overexpression Impairs Patient Survival." *Pigment Cell and Melanoma Research*. https://doi.org/10.1111/pcmr.12249.

Menzel, Moritz, Diana Meckbach, Benjamin Weide, Nora C Toussaint, Karin Schilbach, Seema Noor, Thomas Eigentler, et al. 2014b. "In Melanoma, Hippo Signaling Is Affected by Copy Number Alterations and YAP1 Overexpression Impairs Patient Survival." *Pigment Cell & Melanoma Research* 27 (4): 671–73. https://doi.org/10.1111/pcmr.12249.

Merlino, Glenn, Keith Flaherty, Nicolas Acquavella, Chi-Ping Day, Andrew Aplin, Sheri Holmen, Suzanne Topalian, Terry Van Dyke, and Meenhard Herlyn. 2013. "Meeting Report: The Future of Preclinical Mouse Models in Melanoma Treatment Is Now." *Pigment Cell & Melanoma Research*. https://doi.org/10.1111/pcmr.12099.

Miller, Eric, Jiayi Yang, Michael Deran, Chunlei Wu, Andrew I. Su, Ghislain M.C. Bonamy, Jun Liu, Eric C. Peters, and Xu Wu. 2012. "Identification of Serum-Derived Sphingosine-1-

Phosphate as a Small Molecule Regulator of YAP." *Chemistry and Biology*. https://doi.org/10.1016/j.chembiol.2012.07.005.

Miskolczi, Zsofia, Michael P. Smith, Emily J. Rowling, Jennifer Ferguson, Jorge Barriuso, and Claudia Wellbrock. 2018. "Collagen Abundance Controls Melanoma Phenotypes through Lineage-Specific Microenvironment Sensing." *Oncogene*. https://doi.org/10.1038/s41388-018-0209-0.

Mitra, Satyajit K, and David D Schlaepfer. 2006. "Integrin-Regulated FAK-Src Signaling in Normal and Cancer Cells." *Current Opinion in Cell Biology* 18 (5): 516–23. https://doi.org/10.1016/j.ceb.2006.08.011.

Montagner, Marco, and Sirio Dupont. 2020. "Mechanical Forces as Determinants of Disseminated Metastatic Cell Fate." *Cells*. https://doi.org/10.3390/cells9010250.

Morin-Kensicki, E. M., B. N. Boone, M. Howell, J. R. Stonebraker, J. Teed, J. G. Alb, T. R. Magnuson, W. O'Neal, and S. L. Milgram. 2006. "Defects in Yolk Sac Vasculogenesis, Chorioallantoic Fusion, and Embryonic Axis Elongation in Mice with Targeted Disruption of Yap65." *Molecular and Cellular Biology* 26 (1): 77–87. https://doi.org/10.1128/mcb.26.1.77-87.2006.

Moroishi, Toshiro, Hyun Woo Park, Baodong Qin, Qian Chen, Zhipeng Meng, Steven W. Plouffe, Koji Taniguchi, et al. 2015. "A YAP/TAZ-Induced Feedback Mechanism Regulates Hippo Pathway Homeostasis." *Genes and Development*. https://doi.org/10.1101/gad.262816.115.

Nagano, Makoto, Daisuke Hoshino, Naohiko Koshikawa, Toshifumi Akizawa, and Motoharu Seiki. 2012. "Turnover of Focal Adhesions and Cancer Cell Migration." *International Journal of Cell Biology*. https://doi.org/10.1155/2012/310616.

Nallet-Staub, Flore, Véronique Marsaud, Ling Li, Cristèle Gilbert, Sophie Dodier, Véronique Bataille, Marius Sudol, Meenhard Herlyn, and Alain Mauviel. 2014. "Pro-Invasive Activity of the Hippo Pathway Effectors YAP and TAZ in Cutaneous Melanoma." *Journal of Investigative Dermatology* 134 (1): 123–32. https://doi.org/10.1038/jid.2013.319.

Nguyen, Liem T., Maria S. Tretiakova, Mark R. Silvis, Jared Lucas, Olga Klezovitch, Ilsa Coleman, Hamid Bolouri, et al. 2015. "ERG Activates the YAP1 Transcriptional Program and Induces the Development of Age-Related Prostate Tumors." *Cancer Cell*. https://doi.org/10.1016/j.ccell.2015.05.005.

Niehr, Franziska, Erika von Euw, Narsis Attar, Deliang Guo, Doug Matsunaga, Hooman Sazegar, Charles Ng, et al. 2011. "Combination Therapy with Vemurafenib (PLX4032/RG7204) and Metformin in Melanoma Cell Lines with Distinct Driver Mutations." *Journal of Translational Medicine*. https://doi.org/10.1186/1479-5876-9-76.

Oka, Tsutomu, Eline Remue, Kris Meerschaert, Berlinda Vanloo, Ciska Boucherie, David Gfeller, Gary D. Bader, et al. 2010. “Functional Complexes between YAP2 and ZO-2 Are PDZ Domain-Dependent, and Regulate YAP2 Nuclear Localization and Signalling.” *Biochemical Journal*. https://doi.org/10.1042/BJ20100870.

Oka, Tsutomu, and Marius Sudol. 2009. “Nuclear Localization and Pro-Apoptotic Signaling of YAP2 Require Intact PDZ-Binding Motif.” *Genes to Cells*. https://doi.org/10.1111/j.1365-2443.2009.01292.x.

Olive, Kenneth P., Michael A. Jacobetz, Christian J. Davidson, Aarthi Gopinathan, Dominick McIntyre, Davina Honess, Basetti Madhu, et al. 2009. “Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer.” *Science*. https://doi.org/10.1126/science.1171362.

Oppitz, Matthias, Christian Busch, Gernot Schriek, Marco Metzger, Lothar Just, and Ulrich Drews. 2007. “Non-Malignant Migration of B16 Mouse Melanoma Cells in the Neural Crest and Invasive Growth in the Eye Cup of the Chick Embryo.” *Melanoma Research*. https://doi.org/10.1097/CMR.0b013e3280114f49.

Oudhoff, Menno J., Spencer A. Freeman, Amber L. Couzens, Frann Antignano, Ekaterina Kuznetsova, Paul H. Min, Jeffrey P. Northrop, et al. 2013. “Control of the Hippo Pathway by Set7-Dependent Methylation of Yap.” *Developmental Cell*. https://doi.org/10.1016/j.devcel.2013.05.025.

Palmer, James S., David L. Duffy, Neil F. Box, Joanne F. Aitken, Louise E. O’Gorman, Adele C. Green, Nicholas K. Hayward, Nicholas G. Martin, and Richard A. Sturm. 2000. “Melanocortin-1 Receptor Polymorphisms and Risk of Melanoma: Is the Association Explained Solely by Pigmentation Phenotype?” *American Journal of Human Genetics*. https://doi.org/10.1086/302711.

Paluszczak, Jarosław. 2020. “The Significance of the Dysregulation of Canonical Wnt Signaling in Head and Neck Squamous Cell Carcinomas.” *Cells*. https://doi.org/10.3390/cells9030723.

Panciera, Tito, Luca Azzolin, Michelangelo Cordenonsi, and Stefano Piccolo. 2017. “Mechanobiology of YAP and TAZ in Physiology and Disease.” *Nature Reviews Molecular Cell Biology*. Nature Publishing Group. https://doi.org/10.1038/nrm.2017.87.

Park, Hyun Woo, Young Chul Kim, Bo Yu, Toshiro Moroishi, Jung Soon Mo, Steven W. Plouffe, Zhipeng Meng, et al. 2015. “Alternative Wnt Signaling Activates YAP/TAZ.” *Cell*. https://doi.org/10.1016/j.cell.2015.07.013.

Piccolo, Stefano, Sirio Dupont, and Michelangelo Cordenonsi. 2014. “The Biology of

YAP/TAZ: Hippo Signaling and Beyond." *Physiological Reviews*. https://doi.org/10.1152/physrev.00005.2014.

Plouffe, Steven W., Kimberly C. Lin, Jerrell L. Moore, Frederick E. Tan, Shenghong Ma, Zhen Ye, Yunjiang Qiu, Bing Ren, and Kun Liang Guan. 2018. "The Hippo Pathway Effector Proteins YAP and TAZ Have Both Distinct and Overlapping Functions in the Cell." *Journal of Biological Chemistry* 293 (28): 11230–40. https://doi.org/10.1074/jbc.RA118.002715.

Pobbati, Ajaybabu V., and Wanjin Hong. 2013. "Emerging Roles of TEAD Transcription Factors and Its Coactivators in Cancers." *Cancer Biology and Therapy*. https://doi.org/10.4161/cbt.23788.

Poss, Zachary C., Christopher C. Ebmeier, and Dylan J. Taatjes. 2013. "The Mediator Complex and Transcription Regulation." *Critical Reviews in Biochemistry and Molecular Biology*. https://doi.org/10.3109/10409238.2013.840259.

Prieto, Carlos, and David Barrios. 2019. "RaNA-Seq: Interactive RNA-Seq Analysis from FASTQ Files to Functional Analysis." *Bioinformatics*. https://doi.org/10.1093/bioinformatics/btz854.

Reed, Kurtis B., Jerry D. Brewer, Christine M. Lohse, Kariline E. Bringe, Crystal N. Pruitt, and Lawrence E. Gibson. 2012. "Increasing Incidence of Melanoma among Young Adults: An Epidemiological Study in Olmsted County, Minnesota." *Mayo Clinic Proceedings* 87 (4): 328–34. https://doi.org/10.1016/j.mayocp.2012.01.010.

Remue, Eline, Kris Meerschaert, Tsutomu Oka, Ciska Boucherie, Joël Vandekerckhove, Marius Sudol, and Jan Gettemans. 2010. "TAZ Interacts with Zonula Occludens-1 and -2 Proteins in a PDZ-1 Dependent Manner." *FEBS Letters*. https://doi.org/10.1016/j.febslet.2010.09.020.

Robert, Caroline, Boguslawa Karaszewska, Jacob Schachter, Piotr Rutkowski, Andrzej Mackiewicz, Daniil Stroiakovski, Michael Lichinitser, et al. 2015. "Improved Overall Survival in Melanoma with Combined Dabrafenib and Trametinib." *New England Journal of Medicine*. https://doi.org/10.1056/NEJMoa1412690.

Robert, Caroline, Georgina V. Long, Benjamin Brady, Caroline Dutriaux, Michele Maio, Laurent Mortier, Jessica C. Hassel, et al. 2015. "Nivolumab in Previously Untreated Melanoma without BRAF Mutation." *New England Journal of Medicine*. https://doi.org/10.1056/NEJMoa1412082.

Robles-Espinoza, Carla Daniela, Mark Harland, Andrew J. Ramsay, Lauren G. Aoude, Víctor Quesada, Zhihao Ding, Karen A. Pooley, et al. 2014. "POT1 Loss-of-Function Variants Predispose to Familial Melanoma." *Nature Genetics*. https://doi.org/10.1038/ng.2947.

Rosenbluh, Joseph, Deepak Nijhawan, Andrew G. Cox, Xingnan Li, James T. Neal, Eric J. Schafer, Travis I. Zack, et al. 2012. “β-Catenin-Driven Cancers Require a YAP1 Transcriptional Complex for Survival and Tumorigenesis.” *Cell*. https://doi.org/10.1016/j.cell.2012.11.026.

Rossi, Mariarita, Cristina Pellegrini, Ludovica Cardelli, Valeria Ciciarelli, Lucia di Nardo, and Maria Concetta Fargnoli. 2019. “Familial Melanoma: Diagnostic and Management Implications.” *Dermatology Practical & Conceptual*. https://doi.org/10.5826/dpc.0901a03.

S., Wiley, Sriram K., Liang W., Chang S., McCann T., Nishihara H., French R., and Lowy A. 2018. “GPR68, a Proton Sensing GPCR, Mediates Interaction of Pancreatic Cancer Associated Fibroblasts and Cancer Cells.” *FASEB Journal*.

Saleem, Mohammed D. 2019. “Biology of Human Melanocyte Development, Piebaldism, and Waardenburg Syndrome.” *Pediatric Dermatology*. https://doi.org/10.1111/pde.13713.

Schein, P. S., R. D. Davis, S. Carter, J. Newman, D. R. Schein, and D. P. Rail. 1970. “The Evaluation of Anticancer Drugs in Dogs and Monkeys for the Prediction of Qualitative Toxicities in Man.” *Clinical Pharmacology & Therapeutics*. https://doi.org/10.1002/cpt19701113.

Schiano, Concetta, Amelia Casamassimi, Monica Rienzo, Filomena de Nigris, Linda Sommese, and Claudio Napoli. 2014. “Involvement of Mediator Complex in Malignancy.” *Biochimica et Biophysica Acta - Reviews on Cancer*. https://doi.org/10.1016/j.bbcan.2013.12.001.

Schriek, Gernot, Matthias Oppitz, Christian Busch, Lothar Just, and Ulrich Drews. 2005. “Human SK-Mel 28 Melanoma Cells Resume Neural Crest Cell Migration after Transplantation into the Chick Embryo.” *Melanoma Research*. https://doi.org/10.1097/00008390-200508000-00001.

Shi, Jianxin, Xiaohong R. Yang, Bari Ballew, Melissa Rotunno, Donato Calista, Maria Concetta Fargnoli, Paola Ghiorzo, et al. 2014. “Rare Missense Variants in POT1 Predispose to Familial Cutaneous Malignant Melanoma.” *Nature Genetics*. https://doi.org/10.1038/ng.2941.

Shi, Z., F. He, M. Chen, L. Hua, W. Wang, S. Jiao, and Z. Zhou. 2017. “DNA-Binding Mechanism of the Hippo Pathway Transcription Factor TEAD4.” *Oncogene*. https://doi.org/10.1038/onc.2017.24.

Shimomura, Tadanori, Norio Miyamura, Shoji Hata, Ryota Miura, Jun Hirayama, and Hiroshi Nishina. 2014. “The PDZ-Binding Motif of Yes-Associated Protein Is Required for Its Co-Activation of TEAD-Mediated CTGF Transcription and Oncogenic Cell Transforming Activity.” *Biochemical and Biophysical Research Communications*.

https://doi.org/10.1016/j.bbrc.2013.12.100.

Siegel, Rebecca L., Kimberly D. Miller, and Ahmedin Jemal. 2019. “Cancer Statistics, 2019.” *CA: A Cancer Journal for Clinicians*. https://doi.org/10.3322/caac.21551.

Slemmons, Katherine K., Lisa E.S. Crose, Erin Rudzinski, Rex C. Bentley, and Corinne M. Linardic. 2015. “Role of the YAP Oncoprotein in Priming Ras-Driven Rhabdomyosarcoma.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0140781.

Song, Shumei, Jaffer A. Ajani, Soichiro Honjo, Dipen M. Maru, Qiongrong Chen, Ailing W. Scott, Todd R. Heallen, et al. 2014. “Hippo Coactivator YAP1 Upregulates SOX9 and Endows Esophageal Cancer Cells with Stem-like Properties.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-13-3569.

Soutourina, Julie. 2018. “Transcription Regulation by the Mediator Complex.” *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm.2017.115.

Sudol, Marius. 1994. “Yes-Associated Protein (YAP65) Is a Proline-Rich Phosphoprotein That Binds to the SH3 Domain of the Yes Proto-Oncogene Product.” *Oncogene* 9 (8): 2145–52.

Talantov, Dmitri, Abhijit Mazumder, Jack X Yu, Thomas Briggs, Yuqiu Jiang, John Backus, David Atkins, and Yixin Wang. 2005. “Novel Genes Associated with Malignant Melanoma but Not Benign Melanocytic Lesions.” *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 11 (20): 7234–42. https://doi.org/10.1158/1078-0432.CCR-05-0683.

Tang, Lei, Li Zhou, Shengchun Wu, Xiaoming Shi, Guangwei Jiang, Shuai Niu, and Dianzhu Ding. 2019. “MiR-125a-5p Inhibits Colorectal Cancer Cell Epithelial-Mesenchymal Transition, Invasion and Migration by Targeting TAZ.” *OncoTargets and Therapy* 12: 3481–89. https://doi.org/10.2147/OTT.S191247.

Tang, Zefang, Chenwei Li, Boxi Kang, Ge Gao, Cheng Li, and Zemin Zhang. 2017. “GEPIA: A Web Server for Cancer and Normal Gene Expression Profiling and Interactive Analyses.” *Nucleic Acids Research* 45 (W1): W98–102. https://doi.org/10.1093/nar/gkx247.

Tas, Faruk. 2012. “Metastatic Behavior in Melanoma: Timing, Pattern, Survival, and Influencing Factors.” *Journal of Oncology*. https://doi.org/10.1155/2012/647684.

Tatar, Olcay, Kei Shinoda, Annemarie Adam, Tillmann Eckert, Claus Eckardt, Klaus Lucke, Christoph Deuter, Karl Ulrich Bartz-Schmidt, and Salvatore Grisanti. 2007. “Effect of Verteporfin Photodynamic Therapy on Endostatin and Angiogenesis in Human Choroidal Neovascular Membranes.” *British Journal of Ophthalmology*. https://doi.org/10.1136/bjo.2006.105288.

Terzić, Janoš, and Mirna Saraga-Babić. 1999. “Expression Pattern of PAX3 and PAX6 Genes during Human Embryogenesis.” *International Journal of Developmental Biology*. https://doi.org/10.1387/ijdb.10610023.

Tian, Y., R. Kolb, J.-H. Hong, J. Carroll, D. Li, J. You, R. Bronson, M. B. Yaffe, J. Zhou, and T. Benjamin. 2007. “TAZ Promotes PC2 Degradation through a SCF -Trcp E3 Ligase Complex.” *Molecular and Cellular Biology* 27 (18): 6383–95. https://doi.org/10.1128/mcb.00254-07.

Truebestein, Linda, and Thomas A. Leonard. 2016. “Coiled-Coils: The Long and Short of It.” *BioEssays*. https://doi.org/10.1002/bies.201600062.

Tsai, Kuang Lei, Chieri Tomomori-Sato, Shigeo Sato, Ronald C. Conaway, Joan W. Conaway, and Francisco J. Asturias. 2014. “Subunit Architecture and Functional Modular Rearrangements of the Transcriptional Mediator Complex.” *Cell*. https://doi.org/10.1016/j.cell.2014.05.015.

Tsoi, Jennifer, Lidia Robert, Kim Paraiso, Carlos Galvan, Katherine M. Sheu, Johnson Lay, Deborah J.L. Wong, et al. 2018. “Multi-Stage Differentiation Defines Melanoma Subtypes with Differential Vulnerability to Drug-Induced Iron-Dependent Oxidative Stress.” *Cancer Cell*. https://doi.org/10.1016/j.ccell.2018.03.017.

Uong, Audrey, and Leonard I. Zon. 2010. “Melanocytes in Development and Cancer.” *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.21935.

Varelas, Xaralabos. 2014. “The Hippo Pathway Effectors TAZ and YAP in Development, Homeostasis and Disease.” *Development (Cambridge)*. Company of Biologists Ltd. https://doi.org/10.1242/dev.102376.

Varelas, Xaralabos, Bryan W. Miller, Richelle Sopko, Siyuan Song, Alex Gregorieff, Frederic A. Fellouse, Rui Sakuma, et al. 2010. “The Hippo Pathway Regulates Wnt/β-Catenin Signaling.” *Developmental Cell*. https://doi.org/10.1016/j.devcel.2010.03.007.

Varelas, Xaralabos, Rui Sakuma, Payman Samavarchi-Tehrani, Raheem Peerani, Balaji M. Rao, Joanna Dembowy, Michael B. Yaffe, Peter W. Zandstra, and Jeffrey L. Wrana. 2008. “TAZ Controls Smad Nucleocytoplasmic Shuttling and Regulates Human Embryonic Stem-Cell Self-Renewal.” *Nature Cell Biology*. https://doi.org/10.1038/ncb1748.

Varelas, Xaralabos, Payman Samavarchi-Tehrani, Masahiro Narimatsu, Alexander Weiss, Katie Cockburn, Brett G. Larsen, Janet Rossant, and Jeffrey L. Wrana. 2010. “The Crumbs Complex Couples Cell Density Sensing to Hippo-Dependent Control of the TGF-β-SMAD Pathway.” *Developmental Cell*. https://doi.org/10.1016/j.devcel.2010.11.012.

Velculescu, V. E., S. L. Madden, L. Zhang, A. E. Lash, J. Yu, C. Rago, A. Lal, et al. 1999. "Analysis of Human Transcriptomes [2]." *Nature Genetics*. https://doi.org/10.1038/70487.

Vredeveld, Liesbeth C.W., Patricia A. Possik, Marjon A. Smit, Katrin Meissl, Chrysiis Michaloglou, Hugo M. Horlings, Abderrahim Ajouaou, et al. 2012. "Abrogation of BRAFV600E-Induced Senescence by PI3K Pathway Activation Contributes to Melanomagenesis." *Genes and Development*. https://doi.org/10.1101/gad.187252.112.

Wang, Jun, Yang Xiao, Chih Wei Hsu, Idaliz M. Martinez-Traverso, Min Zhang, Yan Bai, Mamoru Ishii, et al. 2016. "Yap and Taz Play a Crucial Role in Neural Crest-Derived Craniofacial Development." *Development (Cambridge)*. https://doi.org/10.1242/dev.126920.

Wang, Lijuan, Shengjia Shi, Zhangyan Guo, Xiang Zhang, Suxia Han, Angang Yang, Weihong Wen, and Qing Zhu. 2013. "Overexpression of YAP and TAZ Is an Independent Predictor of Prognosis in Colorectal Cancer and Related to the Proliferation and Metastasis of Colon Cancer Cells." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0065539.

Wang, X. Y., X. Jian, B. Q. Sun, X. S. Ge, F. J. Huang, and Y. Q. Chen. 2020. "LncRNA ROR1-AS1 Promotes Colon Cancer Cell Proliferation by Suppressing the Expression of DUSP5/CDKN1A." *European Review for Medical and Pharmacological Sciences*. https://doi.org/10.26355/eurrev_202002_20162.

Webb, Claire, Abhishek Upadhyay, Francesca Giuntini, Ian Eggleston, Makoto Furutani-Seiki, Rieko Ishima, and Stefan Bagby. 2011. "Structural Features and Ligand Binding Properties of Tandem WW Domains from YAP and TAZ, Nuclear Effectors of the Hippo Pathway." *Biochemistry*. https://doi.org/10.1021/bi2001888.

Wei, Honglong, Fuhai Wang, Yong Wang, Tao Li, Peng Xiu, Jingtao Zhong, Xueying Sun, and Jie Li. 2017. "Verteporfin Suppresses Cell Survival, Angiogenesis and Vasculogenic Mimicry of Pancreatic Ductal Adenocarcinoma via Disrupting the YAP-TEAD Complex." *Cancer Science*. https://doi.org/10.1111/cas.13138.

Weiler, Sofia M.E., Teresa Lutz, Michaela Bissinger, Carsten Sticht, Maria Knaub, Norbert Gretz, Peter Schirmacher, and Kai Breuhahn. 2020. "TAZ Target Gene ITGAV Regulates Invasion and Feeds Back Positively on YAP and TAZ in Liver Cancer Cells." *Cancer Letters*. https://doi.org/10.1016/j.canlet.2019.12.044.

Wheatley, Sally P., and Dario C. Altieri. 2019. "Survivin at a Glance." *Journal of Cell Science*. https://doi.org/10.1242/jcs.223826.

Wiesner, Thomas, Anna C. Obenauf, Rajmohan Murali, Isabella Fried, Klaus G. Griewank, Peter Ulz, Christian Windpassinger, et al. 2011. "Germline Mutations in BAP1 Predispose to

Melanocytic Tumors." *Nature Genetics*. https://doi.org/10.1038/ng.910.

Wu, Zhiqiang, Chunmeng Wang, Yong Chen, Zhengwang Sun, and Wangjun Yan. 2020. "SRPX2 Promotes Cell Proliferation and Invasion in Osteosarcoma through Regulating Hippo Signaling Pathway." *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S225602.

Xiong, Tao, and Zeyu Luo. 2018. "The Expression of Actin-Related Protein 2/3 Complex Subunit 5 (ARPC5) Expression in Multiple Myeloma and Its Prognostic Significance." *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research* 24 (September): 6340–48. https://doi.org/10.12659/MSM.908944.

Xu, Jiajie, Pamela J. Vanderzalm, Michael Ludwig, Ting Su, Sherzod A. Tokamov, and Richard G. Fehon. 2018. "Yorkie Functions at the Cell Cortex to Promote Myosin Activation in a Non-Transcriptional Manner." *Developmental Cell*. https://doi.org/10.1016/j.devcel.2018.06.017.

Xu, Man, Yongle Li, Jinfeng Du, Hengrong Lin, Sixian Cao, Zuming Mao, Ronghua Wu, Mei Liu, Yan Liu, and Qiyou Yin. 2018. "PAX3 Promotes Cell Migration and CXCR4 Gene Expression in Neural Crest Cells." *Journal of Molecular Neuroscience*. https://doi.org/10.1007/s12031-017-0995-9.

Yagi, Ryohei, Lin Feng Chen, Katsuya Shigesada, Yota Murakami, and Yoshiaki Ito. 1999. "A WW Domain-Containing Yes-Associated Protein (YAP) Is a Novel Transcriptional Co-Activator." *EMBO Journal* 18 (9): 2551–62. https://doi.org/10.1093/emboj/18.9.2551.

Yang, Hong, Brian Higgins, Kenneth Kolinsky, Kathryn Packman, Zenaida Go, Raman Iyer, Stanley Kolis, et al. 2010. "RG7204 (PLX4032), a Selective BRAFV600E Inhibitor, Displays Potent Antitumor Activity in Preclinical Melanoma Models." *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-10-0646.

Yin, Jing Wen, and Gang Wang. 2014. "The Mediator Complex: A Master Coordinator of Transcription and Cell Lineage Development." *Development (Cambridge)*. https://doi.org/10.1242/dev.098392.

Yokoyama, Satoru, Susan L. Woods, Glen M. Boyle, Lauren G. Aoude, Stuart MacGregor, Victoria Zismann, Michael Gartside, et al. 2011. "A Novel Recurrent Mutation in MITF Predisposes to Familial and Sporadic Melanoma." *Nature*. https://doi.org/10.1038/nature10630.

You, Faping, Junhui Li, Peijin Zhang, Hui Zhang, and Xuchen Cao. 2020. "MiR106a Promotes the Growth of Transplanted Breast Cancer and Decreases the Sensitivity of Transplanted Tumors to Cisplatin." *Cancer Management and Research*.

https://doi.org/10.2147/CMAR.S231375.

Yu, Fa Xing, Jing Luo, Jung Soon Mo, Guangbo Liu, Young Chul Kim, Zhipeng Meng, Ling Zhao, et al. 2014. “Mutant Gq/11 Promote Uveal Melanoma Tumorigenesis by Activating YAP.” *Cancer Cell*. https://doi.org/10.1016/j.ccr.2014.04.017.

Yu, Fa Xing, Bin Zhao, Nattapon Panupinthu, Jenna L. Jewell, Ian Lian, Lloyd H. Wang, Jiagang Zhao, et al. 2012. “Regulation of the Hippo-YAP Pathway by G-Protein-Coupled Receptor Signaling.” *Cell*. https://doi.org/10.1016/j.cell.2012.06.037.

Yuan, Yanli, Deyu Li, Haibo Li, Liancai Wang, Guangjin Tian, and Yadong Dong. 2016. “YAP Overexpression Promotes the Epithelial-Mesenchymal Transition and Chemoresistance in Pancreatic Cancer Cells.” *Molecular Medicine Reports* 13 (1): 237–42. https://doi.org/10.3892/mmr.2015.4550.

Zaidi, Sayyed K., Andrew J. Sullivan, Ricardo Medina, Yoshiaki Ito, Andre J. Van Wijnen, Janet L. Stein, Jane B. Lian, and Gary S. Stein. 2004. “Tyrosine Phosphorylation Controls Runx2-Mediated Subnuclear Targeting of YAP to Repress Transcription.” *EMBO Journal*. https://doi.org/10.1038/sj.emboj.7600073.

Zanconato, Francesca, Giusy Battilana, Mattia Forcato, Letizia Filippi, Luca Azzolin, Andrea Manfrin, Erika Quaranta, et al. 2018. “Transcriptional Addiction in Cancer Cells Is Mediated by YAP/TAZ through BRD4.” *Nature Medicine* 24 (10): 1599–1610. https://doi.org/10.1038/s41591-018-0158-8.

Zanconato, Francesca, Michelangelo Cordenonsi, and Stefano Piccolo. 2016a. “YAP/TAZ at the Roots of Cancer.” *Cancer Cell*. https://doi.org/10.1016/j.ccell.2016.05.005.

Zanconato, Francesca, Mattia Forcato, Giusy Battilana, Luca Azzolin, Erika Quaranta, Beatrice Bodega, Antonio Rosato, Silvio Bicciato, Michelangelo Cordenonsi, and Stefano Piccolo. 2015a. “Genome-Wide Association between YAP/TAZ/TEAD and AP-1 at Enhancers Drives Oncogenic Growth.” *Nature Cell Biology*. https://doi.org/10.1038/ncb3216.

Zbytek, Blazej, J. Andrew Carlson, Jacqueline Granese, Jeffrey Ross, Martin Mihm, and Andrzej Slominski. 2008. “Current Concepts of Metastasis in Melanoma.” *Expert Review of Dermatology*. https://doi.org/10.1586/17469872.3.5.569.

Zhang, Haiying, H Amalia Pasolli, and Elaine Fuchs. 2011. “Yes-Associated Protein (YAP) Transcriptional Coactivator Functions in Balancing Growth and Differentiation in Skin.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (6): 2270–75. https://doi.org/10.1073/pnas.1019603108.

Zhang, Heng, Chen Ying Liu, Zheng Yu Zha, Bin Zhao, Jun Yao, Shimin Zhao, Yue Xiong, Qun

Ying Lei, and Kun Liang Guan. 2009. “TEAD Transcription Factors Mediate the Function of TAZ in Cell Growth and Epithelial-Mesenchymal Transition.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M900843200.

Zhang, Huabing, Sadeesh K. Ramakrishnan, Daniel Triner, Brook Centofanti, Dhiman Maitra, Balázs Gyorffy, Judith S. Sebolt-Leopold, et al. 2015. “Tumor-Selective Proteotoxicity of Verteporfin Inhibits Colon Cancer Progression Independently of YAP1.” *Science Signaling*. https://doi.org/10.1126/scisignal.aac5418.

Zhang, Wenjing, Yijun Gao, Peixue Li, Zhubing Shi, Tong Guo, Fei Li, Xiangkun Han, et al. 2014. “VGLL4 Functions as a New Tumor Suppressor in Lung Cancer by Negatively Regulating the YAP-TEAD Transcriptional Complex.” *Cell Research*. https://doi.org/10.1038/cr.2014.10.

Zhang, Xiaomeng, Jian Zhong Tang, Ismael A. Vergara, Youfang Zhang, Pacman Szeto, Lie Yang, Christopher Mintoff, et al. 2019. “Somatic Hypermutation of the YAP Oncogene in a Human Cutaneous Melanoma.” *Molecular Cancer Research* 17 (7): 1435–49. https://doi.org/10.1158/1541-7786.MCR-18-0407.

Zhang, Yinglong, He Shen, Henry G. Withers, Nuo Yang, Kayla E. Denson, Ashley L. Mussell, Alexander Truskinovsky, et al. 2017. “VGLL4 Selectively Represses YAP-Dependent Gene Induction and Tumorigenic Phenotypes in Breast Cancer.” *Scientific Reports*. https://doi.org/10.1038/s41598-017-06227-7.

Zhang, Yu Hang, Dong Dong Luo, Sheng Biao Wan, and Xian Jun Qu. 2020. “S1PR2 Inhibitors Potently Reverse 5-FU Resistance by Downregulating DPD Expression in Colorectal Cancer.” *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2020.104717.

Zhao, Bin, Xiaomu Wei, Weiquan Li, Ryan S. Udan, Qian Yang, Joungmok Kim, Joe Xie, et al. 2007. “Inactivation of YAP Oncoprotein by the Hippo Pathway Is Involved in Cell Contact Inhibition and Tissue Growth Control.” *Genes and Development*. https://doi.org/10.1101/gad.1602907.

Zhao, Bin, Xin Ye, Jindan Yu, Li Li, Weiquan Li, Siming Li, Jianjun Yu, et al. 2008a. “TEAD Mediates YAP-Dependent Gene Induction and Growth Control.” *Genes and Development*. https://doi.org/10.1101/gad.1664408.

Zuo, L., J. Weger, Q. Yang, A. M. Goldstein, M. A. Tucker, G. J. Walker, N. Hayward, and N. C. Dracopoli. 1996. “Germline Mutations in the P16(INK4a) Binding Domain of CDK4 in Familial Melanoma.” *Nature Genetics*. https://doi.org/10.1038/ng0196-97.